Reactivities of indoleamine 2,3-dioxygenases

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2013

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Abstract

The heme enzyme indoleamine 2,3-dioxygenase (IDO1) catalyzes L-Trp oxidation in non-hepatic mammalian tissues by inserting both atoms of dioxygen into the indole ring to form N-formylkynurenine.

In the present work, IDO1 was found to oxidize β-NADH under aerobic conditions in the absence of other reactants. This reaction led to formation of the reactive dioxygen-adduct of this enzyme and supported the oxidation of L-Trp to N-formylkynurenine. These processes were accelerated by hydrogen peroxide, and inhibited by either superoxide dismutase or catalase. In contrast, anaerobic reaction of IDO1 with β-NADH required an electron-transfer mediator. It is proposed that trace amounts of peroxide formed by reaction of dioxygen with β-NADH lead to one-electron oxidation of this substrate by IDO1, whose products reduce dioxygen to superoxide which in turn reacts with the enzyme to form the dioxygen-adduct. Regeneration of peroxide through auto-oxidation of the dioxygen-adduct of IDO1 results in auto-catalytic formation of this enzyme product in the presence of β-NADH.

IDO1 was also found to catalyze the oxidation of indole by hydrogen peroxide, both of which were independently thought to be non-substrate, and non-oxygen-donor, respectively. This reaction resulted in O₂-independent formation of monooxygenated indoles and was not inhibited by superoxide dismutase or hydroxyl radical scavengers. Consumption of indole and peroxide exhibited a near one-to-one correspondence and isotopic labeling experiments identified peroxide as the source of oxygen incorporated by indole oxidation products. It is proposed that indole oxidation was coupled to oxygen transfer from ferryl species of IDO1 formed by reaction with peroxide by means of a peroxygenase activity that is mechanistically analogous to the peroxide-shunt activation of cytochrome P450 enzymes.
A bacterial expression system for a human isozyme of IDO1 (IDO2) was constructed. Despite similar electronic properties to IDO1, recombinant IDO2 was far less efficient at catalyzing L-Trp oxidation. The midpoint reduction potentials and auto-oxidation kinetics of IDO2 were comparable to those of IDO1, arguing against enzyme oxidation as a significant contributor of diminished enzyme activity. It is proposed that differences in L-Trp binding properties of the isozymes are mainly responsible for this catalytic disparity.
Preface

Parts of this dissertation have been published in peer-reviewed journals.

Chapter 3, section 3.1 and Chapter 4, section 4.1 are based on work conducted in close collaboration with Dr. F. I. Rosell. In this study, Dr. F. I. Rosell and I produced the enzyme. I designed and performed the experiments that evaluate the effects of L-Trp on \( \beta \)-NADH-oxidase activity and the associated L-Trp dioxygenase activity of the enzyme. Dr. F. I. Rosell wrote the larger part of the manuscript, and I wrote sections associated with my experiments.

A version of Chapter 3, section 3.1 and Chapter 4, section 4.1 has been published in the *Journal of Biological Chemistry* (Rosell FI, Kuo HH, Mauk AG (2011) NADH oxidase activity of indoleamine 2,3-dioxygenase. *J Biol Chem* 286:29273-83).

Chapter 3, section 3.2 and Chapter 4, section 4.2 are based on work I conducted. In this study, I was responsible for producing enzyme, designing and performing experiments in characterizing the indole peroxygenase activity of the enzyme, and writing the manuscript.

A version of Chapter 3, section 3.2 and chapter 4, section 4.2 has been published in the *Proceedings of the National Academy of Sciences* (Kuo HH, Mauk AG (2012) Indole peroxygenase activity of indoleamine 2,3-dioxygenase. *P Natl Acad Sci USA* 109:13966-71).
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# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>δ-ALA</td>
<td>δ-Aminolevulinic acid</td>
</tr>
<tr>
<td>1MT</td>
<td>1-Methyl-D/L-tryptophan</td>
</tr>
<tr>
<td>3-OH-kyn</td>
<td>3-hydroxykynurenine</td>
</tr>
<tr>
<td>3-OH-kynG</td>
<td>3-hydroxykynurenine glucoside</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>AHBG</td>
<td>4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid glucoside</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>Bis-Tris</td>
<td>Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane</td>
</tr>
<tr>
<td>CHES</td>
<td>2-(Cyclohexylamino)ethanesulfonic acid</td>
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<td>CT</td>
<td>Charge transfer</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ENDOR</td>
<td>Electron nuclear double resonance</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>FAD⁺</td>
<td>Flavin adenine dinucleotide (oxidized)</td>
</tr>
<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>LUMO</td>
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<td>Acronym</td>
<td>Description</td>
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<td>NADH</td>
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<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
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<td>NMDA</td>
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<td>Nicotinamide mononucleotide (reduced)</td>
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<td>NOR</td>
<td>Nitric oxide reductase</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PIM</td>
<td>4-Phenylimidazole</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>QM/MM</td>
<td>Quantum mechanic /molecular mechanic</td>
</tr>
<tr>
<td>RMSD</td>
<td>Root-mean-square-deviation</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TAPS</td>
<td>[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid</td>
</tr>
<tr>
<td>TDLN</td>
<td>Tumor draining lymph nodes</td>
</tr>
<tr>
<td>TDO</td>
<td>L-Tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>TEV</td>
<td>Tabacco etch virus</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
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<td>Ultraviolet</td>
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</tbody>
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Acknowledgements

First and foremost, I would like to express my sincere respect and deepest gratitude to my advisor, Grant Mauk, who has supported me throughout my thesis with his patience and knowledge while allowing me the freedom to pursue my own ideas. His guidance over these past five years has been indispensable to my growth as a scientist.

I am deeply indebted to many of my colleagues, past and present members of the Mauk lab, for their kindness, friendship, and guidance. Thank you Marcia, Joseph, Tomoko, Marco, Rajesh, Tamara, and Anne. I want to especially thank Fred and Steve for their insightful discussions and continuous assistance during this research. I could not have asked for better friends. It has been a privilege working with you guys.

Thanks go out to my committee members, Michael Murphy and Michel Roberge, for devoting their time and energy in reviewing this thesis.

Finally, I thank my parents for their constant support and encouragement. This work is your accomplishment as much as it is mine.
Chapter 1: introduction

1.1 Overview

1.1.1 Dioxygen

In 1755, the English chemist Joseph Priestly observed that air is not an elementary substance as Aristotle had proposed centuries before but that it is a composition that in its purest form is “dephlogisticated air”—or as the French chemist Antoine Lavoisier who later named it—“oxygen” (Priestly 1775). Priestly’s revelation formed the experimental basis of Lavoisier’s oxidation chemistry.

Molecular oxygen, or dioxygen (O$_2$), is a powerful oxidant. The four one-electron reduction reactions of O$_2$ are exothermic and yield in this order: superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (HO$^*$), and finally water (Ingraham & Meyer 1985). Even though oxidation of organic compounds by O$_2$ is thermodynamically favoured, these reactions are slow at ambient temperature. This apparent dichotomy in O$_2$ reactivity is attributable to its electronic structure at the ground state. Whereas stable organic compounds generally occur in nature in a closed-shell state (i.e., singlet, $S=0$), O$_2$ has a triplet ground state (i.e., $S=1$) that results from the two unpaired electrons in the two degenerate anti-bonding orbitals (i.e., ($\pi_x^*2p$)$_1$, ($\pi_y^*2p$)$_1$) (Ingraham & Meyer 1985, Traylor & Traylor 1995). As a result of this electronic configuration, ionic reactions with O$_2$ require that O$_2$ changes spin state before the two spin-paired electrons of the substrate can occupy the unfilled orbitals. This triplet-singlet transition is spin-forbidden quantum mechanically as a result of the large activation barrier associated with spin inversion, and, therefore, occurs at a rate that is slow relative to the lifetime of a collision complex. Consequently, oxidation reactions of triplet O$_2$ are generally limited to free radicals (i.e., doublets) (Sono et al. 1996). Conversely, triplet O$_2$ can be converted to a singlet (i.e., ($\pi_x^*2p$)$_2$, ($\pi_y^*2p$)$_0$) if given sufficient activation energy, generally via energy
transfer from organic molecules in their excited states (DeRosa & Crutchley 2002). A corollary of the loss in the degeneracy of the anti-bonding orbitals is that singlet \( \text{O}_2 \) is far more reactive than triplet \( \text{O}_2 \) because its unfilled \( \pi^* \) orbital readily accepts paired electrons. In biological systems, activation of triplet \( \text{O}_2 \) is generally mediated by reactions with transition metals (Sono \textit{et al.} 1996). For example, orbital mixing between iron and triplet \( \text{O}_2 \) can result in a new electronic configuration that renders \( \text{O}_2 \) singlet-like in nature. Metal-oxygen complexes in metallo-proteins are thus able to support a wide-array of biological oxidations.

### 1.1.2 Oxygenases

Throughout the early 20\textsuperscript{th} century, the dehydrogenation theory advocated by Heinrich Wieland had been the dogma of biological oxidations. According to Wieland, \( \text{O}_2 \) in biological systems serves as an electron and hydrogen acceptor, but it is not incorporated directly into metabolites (Wieland 1932). In 1955 however, Osamu Hayaishi and Howard Mason independently demonstrated the incorporation of \( \text{O}_2 \) into catechol and 3,4-dimethylphenol by iron-containing catechol dioxygenase (Hayaishi \textit{et al.} 1955) and copper-containing phenolase (Mason \textit{et al.} 1955), respectively. The discovery of \( \text{O}_2 \) as a substrate for these enzymes resulted in identification of a new class of metallo-enzymes that was given the name “oxygenase”. Today two general classes of oxygenase are recognized: monooxygenases that incorporate one atom of oxygen per molecule of \( \text{O}_2 \) into the substrate (reaction 1.1), and dioxygenases that incorporate both atoms of oxygen from \( \text{O}_2 \) into the substrate (reaction 1.2).

\[
\text{RH} + \text{O}_2 + 2e^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} \quad (1.1)
\]

\[
\text{R} + \text{O}_2 \rightarrow \text{RO}_2 \quad (1.2)
\]

Iron is the transition metal most commonly present in the hundreds of oxygenases that are now known. Iron centres found in oxygenases occur as mononuclear iron, binuclear iron, iron-sulfur clusters, and heme (iron protoporphyrin IX), or other iron-containing tetrapyrroles. Each
of these types of iron centre has been found at the active sites of both monooxygenases and dioxygenases (**Table 1.1**) (Que & Ho 1996, Sono *et al.* 1996, Wallar & Lipscomb 1996, Fitzpatrick 2000, Wackett 2002, Hausinger 2004). Whereas monooxygenases are represented by the very large family of P450 cytochromes (currently > 2000 members) that possess an active-site heme, most dioxygenases contain mono-nuclear iron sites, sometimes in combination with iron-sulfur clusters. In comparison, heme-containing dioxygenases are far less common than are heme-containing monooxygenase. Enzymes that have been identified in this family so far include prostaglandin H synthase (Karthein *et al.* 1988), linoleate 8-dioxygenase (Hamberg *et al.* 1994), fatty acid α-dioxygenase (Hamberg *et al.* 2005), L-tryptophan 2,3-dioxygenase, and indoleamine 2,3-dioxygenase (Feigelson & Brady 1973, Hayaishi 1976). The first three members of this family carry out one electron oxidations of their respective fatty acid substrates in a heme-dependent manner to the corresponding free radicals, which subsequently incorporate free O₂ (Karthein *et al.* 1988, Hamberg *et al.* 1994, Hamberg *et al.* 2005). In contrast, the last two members of this family, L-tryptophan- and indoleamine 2,3-dioxygenase, incorporate heme-bound O₂ into the substrate (Feigelson & Brady 1973, Hayaishi 1976, Hayaishi *et al.* 1977). In his early studies of these two heme-containing dioxygenases, Hayashi proposed that activated O₂ is involved in a ternary complex of heme:oxygen:substrate (Hirata *et al.* 1977), an hypothesis that was later substantiated for the broader family of heme-containing monooxygenases as well (Gunsalus *et al.* 1968, Estabrook *et al.* 1971). The ability of these enzymes to incorporate one or both atoms of dioxygen into their substrates amply demonstrates the catalytic versatility of heme-containing enzymes.

L-Tryptophan- and indoleamine 2,3-dioxygenase (TDO and IDO, respectively) are involved in the catabolism of L-Trp and catalyze essentially the same reaction: insertion of O₂ into L-Trp to yield N-formylkynurenine (N-FK) (**Figure 1.1**). Chapter 1 reviews the literature primarily concerning IDO.
<table>
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<th>Monooxygenases</th>
<th>Dioxygenases</th>
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<td>Heme</td>
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<td>L-Trp 2,3-dioxygenase</td>
</tr>
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<td>Secondary amine monooxygenases</td>
<td>Indoleamine 2,3-dioxygenase</td>
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<td>Heme oxygenases</td>
<td>Prostaglandin H synthase</td>
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<table>
<thead>
<tr>
<th>Properties</th>
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<th>TDO</th>
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<tbody>
<tr>
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<td>1</td>
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</tr>
<tr>
<td>Oxygen source</td>
<td>O₂ and O₂²⁻</td>
<td>O₂</td>
</tr>
<tr>
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<td>L- and D-Trp, L- and D-5-hydroxy-Trp, tryptamine, serotonin.</td>
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</tr>
<tr>
<td>Kₘ (µM)</td>
<td>15 for L-Trp, 2600 for D-Trp</td>
<td>190 for L-Trp, 180 for D-Trp</td>
</tr>
<tr>
<td>k₉ (s⁻¹)</td>
<td>3.1 for L-Trp, 5.9 for D-Trp</td>
<td>2.1 for L-Trp, 0.2 for D-Trp</td>
</tr>
<tr>
<td>Tissue distribution</td>
<td>Ubiquitous (except liver)</td>
<td>Liver</td>
</tr>
<tr>
<td>Inducers</td>
<td>Interferon-γ, influenza, HIV virus, lipopolysaccharides.</td>
<td>L-Trp, hydrocortisone.</td>
</tr>
</tbody>
</table>

References: 1, (Sono et al. 1996); 2, (Karthein et al. 1988); 3, (Hamberg et al. 1994); 4, (Hamberg et al. 2005); 5, (Fitzpatrick 2000); 6, (Hausinger 2004); 7, (Que & Ho 1996); 8, (Waller & Lipscomb 1996); 9, (Wackett 2002).

Table 1.2 Comparison of the biochemical properties of human indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) *

* Adapted from (Sono et al. 1996). References: 1, (Lu et al. 2009); 2, (Batabyal & Yeh 2007).
Figure 1.1 Dioxygenase activity of tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO). TDO- and IDO-catalyzed conversion of L-Trp to N-formylkynurenine (N-FK) by the insertion of both atoms of O2 at the C2 and C3 positions of the indole ring and concomitant with cleavage of the C2–C3 π-bond.

1.2 L-Trp catabolism and the kynurenine pathway

Tryptophan (L-Trp) is the least abundant of the essential amino acids. In addition to being required for protein synthesis, L-Trp is a key precursor for the biosynthesis of the neurotransmitter serotonin. Approximately 1% of dietary L-Trp is hydroxylated by L-Trp 5-hydroxylase in the brain (Zhang et al. 2004) and in the pineal gland (Patel et al. 2004) to serotonin. In the pineal gland, serotonin is further converted to the hormone melatonin (Ganguly
The remaining 95% of L-Trp metabolism occurs through the kynurenine pathway in which IDO and TDO catalyze the first step (Takikawa 2005). This pathway leads to the complete oxidation of the L-Trp carbon skeleton and in the process provides key precursors for the biosynthesis of the cofactor nicotinamide adenine dinucleotide (NAD) and of the neuroactive compound quinolinic acid. These pathways are shown schematically in Figure 1.2.

1.2.1 The gate-keepers of the kynurenine pathway

TDO and IDO both catalyze the first step in the kynurenine pathway, oxidation of L-Trp to N-FK, and so determine how much L-Trp is processed by this route. Although they are both cytosolic heme proteins catalyzing the same reaction, they differ in a number of basic properties (Table 1.2). IDO is a monomeric enzyme (M.W. 45 kDa) whereas TDO is tetrameric (protomer M.W. 48 kDa). The two proteins are antigenically distinct and do not share significant sequence identity or even global structural similarity (Sugimoto et al. 2006, Forouhar et al. 2007). Also, neither IDO nor TDO share significant sequence identity with other mammalian heme proteins such as hemoglobin, myoglobin, and cytochromes P450 although gastropod myoglobins are reportedly evolutionarily related to mammalian IDO (Suzuki et al. 1998). Mammalian TDO is expressed mainly in the liver (Maezono et al. 1990), whereas IDO is expressed and regulates L-Trp metabolism in all extra-hepatic tissues (Hayaishi et al. 1975, Yamazaki et al. 1985). TDO activity is induced specifically by L-Trp, non-specifically by corticosteroids (Greengard & Feigelson 1961, Altman & Greengard 1966), and suppressed by metabolites of the kynurenine pathway (Bender 1983). As such, TDO appears to be primarily involved in regulating dietary and systemic L-Trp concentrations. On the other hand, IDO activity increases during endotoxic shock or viral infections (Yoshida et al. 1979) and correlates directly with the level of interferon-γ (Takikawa et al. 1988), a pro-inflammatory cytokine that plays critical regulatory roles in the innate and adaptive immune systems (Billiau 1996, Tau & Rothman 1999, Muhl & Pfeilschifter 2003). Although human TDO and IDO catalyze L-Trp oxidation at comparable rates
Figure 1.2 Mammalian tryptophan metabolic pathways.
(Batabyal & Yeh 2007), there is a notable difference in the two enzymes with respect to substrate recognition. TDO is highly specific towards L-Trp (Tanaka & Knox 1959, Watanabe et al. 1980). Rabbit IDO, however, is active towards L- and D-Trp, L- and D-5-hydroxy-Trp, tryptamine, and serotonin, and human IDO is active towards all of these substrates except serotonin (Takikawa et al. 1988).

1.2.2 Tryptophan and kynurenine imbalance

There is a long history of efforts to identify pathology associated with abnormal L-Trp metabolism. Many of these clinical reports indicate elevated levels of kynurenine and related metabolites in urine and/or serum of individuals afflicted with rheumatoid arthritis (Labadarios et al. 1978), schizophrenia (Erhardt et al. 2001), porphyria (Litman & Correia 1983), aplastic anemia (Altman & Greengard 1966), acquired immunodeficiency (AIDS) dementia complex, amyotrophic lateral sclerosis, the syndromes of Alzheimer, Huntington, and Parkinson (Beal et al. 1990, Guillemin & Brew 2002, Bruijn et al. 2004, Guillemin et al. 2005), and several types of cancer, including Hodgkin’s lymphoma, colorectal and gynecological cancer (Brown et al. 1960, De Vita et al. 1971, Brandacher et al. 2006). The etiological significance of kynurenine metabolites in some of these diseases (i.e., cancer and neurodegenerative disorders) is discussed in sections 1.3.1 and 1.3.2.

In the past, these clinical conditions were often interpreted as resulting from a functional deficiency in pyridoxine (Brown et al. 1961, Korbitz et al. 1963), the precursor to pyridoxal phosphate, which is required for kynureninase and kynurenine transaminase activities (Knox 1953). Indeed, pyridoxine supplementation was shown in some cases (i.e., rheumatoid arthritis and Hodgkin’s lymphoma) to correct the disordered metabolic pattern (Bett 1962, Allegri et al. 1972). Altman, however, noted that it is unlikely that the variety of cancers and diseases associated with abnormalities in L-Trp metabolism were coupled solely to pyridoxine deficiency (Altman & Greengard 1966). It was further suggested that the observed changes in kynurenine
metabolite levels were probably not disease-specific; these changes were instead re-interpreted as the result of metabolic response to illness-induced stress (Rose 1972), consistent with the induction of TDO activity by release of adrenal corticosteroids (Altman & Greengard 1966). The subsequent finding that IDO activity is induced by interferon-\(\gamma\) (Takikawa et al. 1988) enabled similar re-interpretation of these older observations in the context of immune response, especially those pertaining to cancer-induced L-Trp metabolic abnormalities as discussed in section 1.3.3.

1.3 Biological roles of IDO

In recent years, the literature concerning the biological roles of IDO has grown rapidly, and several medical implications for IDO activity have been identified or proposed (Takikawa 2005, Muller & Prendergast 2007). Some of the pathological states associated with imbalance of L-Trp and kynurenine, as discussed earlier, appear to result from failure in the regulatory mechanism of IDO. Specifically, the accumulation of metabolites from the kynurenine pathway (i.e., kynurenine, 3-hydroxyantrhanilic acid, and quinolinic acid) in non-hepatic tissues has been linked to ocular, neurological and immunological disorders (Takikawa et al. 2003, Takikawa 2005).

1.3.1 Kynurenines and senile-cataracts

Senile cataracts are characterized by the opacification and hardening of the human lens and are the most common causes of vision loss worldwide (Asbell et al. 2005). Aging is the primary risk factor although other factors have been suggested as well (i.e., smoking, diabetes, obesity, sunlight overexposure). The human lens contains filter compounds that absorb strongly in the ultraviolet (UV) and near-UV regions of the electromagnetic spectrum to protect the retina against UV-induced damage and to enhance visual acuity (Korlimbinis & Truscott 2006). These compounds include kynurenine, 3-hydroxykynurenine (3-OH-kyn), 3-hydroxykynurenine
glucoside (3-OH-kynG), 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid glucoside (AHBG), and glutathionyl-3-hydroxykynurenine glucoside (Streete et al. 2004), all of which are intermediates in the kynurenine pathway and result from degradation of L-Trp. Despite their protective properties, many of these filter compounds are oxidized slowly to form H₂O₂ (Vazquez et al. 2000) and to cause oxidative damage to crystallins (lens protein). Korlimbinis and co-workers have demonstrated that 3-OH-kyn catalyzes the oxidation of methionine residues of crystallins in vitro, which could contribute to the covalent cross-linking and insolubilization of crystallins associated with senile cataract formation (Korlimbinis & Truscott 2006). Aging has also been shown to correlate with formation of kynurenine-, 3-OH-kyn-, and 3-OH-kynG-adducts of crystallin (Aquilina et al. 1997, Hood et al. 1999), a process known as kynurenilation. The levels of free 3-OH-kynG and downstream metabolites are reduced correspondingly in cataract lens as compared to normal lens, whereas levels of L-Trp are higher in cataract lens (Streete et al. 2004). These crystallin-adducts are yellow and fluorescent, and because there is little to no crystallin turnover in the lens (Bloemendal 1977), kynurenilation results in gradual discolouration of the lens.

IDO, being the enzyme that catalyzes the rate-limiting step in the kynurenine pathway, has been speculated to play a role in the pathogenesis of senile-cataracts. IDO activity is detected in lenses ranging from 26 to 80 years, and changes little with age (Takikawa et al. 2001). Moreover, IDO expression is induced in corneal cells by exposure to UV light, presumably as a protective mechanism (Serbecic & Beutelspacher 2006). Currently, surgical removal of the opacified lens is the only treatment available for senile-cataracts. Although therapeutic intervention of IDO activity is unlikely to restore visual acuity in deteriorated lenses, it has been proposed that such treatment could help reduce the levels of these reactive UV filters, prevent oxidative modification of the lens proteins (Takikawa 2005), and thereby slow the development of senile-cataracts.
1.3.2 Quinolinic acid and neuro-degenerative diseases

Quinolinic acid is an agonist for the neuronal $N$-methyl-$d$-aspartic acid (NMDA) subtype of glutamate receptors (Stone & Perkins 1981) which plays a role in synaptic plasticity and long-term potentiation mechanisms that are believed to be important for learning and memory (Morris et al. 1986, Lee & Silva 2009). The levels of quinolinic acid are elevated in individuals afflicted with ALS, syndromes of Alzheimer, Huntington, and Parkinson (Guillemin et al. 2005), as well as in patients suffering from inflammatory neurologic disorders including AIDS dementia complex, poliomyelitis (Heyes et al. 1992), and cerebral malaria (Sanni et al. 1998). Intracerebral administration of quinolinic acid has been shown to cause neuronal cell death (Schwarcz et al. 1983), and similar results have been obtained by treatment of cultures of astrocytes and oligodendrocytes with quinolinic acid (Cammer 2002, Guillemin et al. 2005). The mechanism of cellular destruction by quinolinic acid has not been completely elucidated, but appears to be related to accumulation of reactive oxygen species resulting from excessive activation of NMDA receptors (Braidy et al. 2009).

Endogenous levels of quinolinic acid in the central nervous system are normally low compared to those in systemic tissues, but these increase dramatically during an inflammatory response (Heyes et al. 1992, Flanagan et al. 1995). Because the increase in quinolinic concentrations closely reflects the local IDO activity (Heyes et al. 1991), it has been suggested that inflammation-induced up-regulation of IDO expression in the brain results in accumulation of excess quinolinic acid that promotes pathogenesis of neurological disorders (Takikawa 2005). On the other hand, kynurenic acid and picolinic acid are also metabolites of the kynurenine pathway, but they are antagonists of NMDA receptors and so exhibit neuroprotective properties. Kynurenic acid and picolinic acid are synthesized by kynurenine transaminase and aminocarboxymuconate semialdehyde decarboxylase, respectively (Mason 1954, Pucci et al. 2007). It is likely that additional regulatory mechanisms for these enzymes direct the kynurenine pathway.
pathway between the production of neuroprotectants and neurotoxins. Interestingly, Guillemin and co-workers report a reduction in TDO expression when IDO expression is increased in neurons and _vice versa_ that led the authors to suggest the existence of a regulatory mechanism that balances kynurenine metabolite production between these two enzymes (Guillemin _et al._ 2007).

1.3.3 IDO and immunosuppression

In the early 20th century, Paul Ehrlich first suggested that the immune system detects and eradicates nascent transformed cells that would otherwise accumulate continuously (Ehrlich 1909). Nearly a century later, the concept of immune surveillance has evolved further to include the concepts of an immune equilibrium and immune escape (Dunn _et al._ 2002). Immune equilibrium is a stage in which tumor cells continue to divide while being contained by the immune system, in the process creating cellular pressure that selects for tumor cells that are able to elude destruction. Immune escape is a stage in which tumor cells have evolved mechanisms that allow them to evade and suppress hostile elements of the immune system to result in appearance of clinical manifestations of the disease.

Emerging evidence indicates that activation of IDO and the kynurenine pathway suppresses the functions of the immune system (Munn _et al._ 1998, Munn _et al._ 1999, Mellor & Munn 2004, Takikawa 2005), and therefore suggests that IDO may play a role in facilitating the transition from tumor immune equilibrium to immune escape. Cell growth is regulated by local nutrient concentrations such that even slight deviation from controlled concentrations can alter cell functions or even trigger cell cycle arrest. Specifically, the proliferation of T-lymphocytes that maintain host immunity is particularly sensitive to the availability of L-Trp (Lee _et al._ 2002). Macrophages, tumor cells and a subset of dendritic cells expressing IDO can deplete their local microenvironment of L-Trp, causing T-lymphocytes to enter cell cycle arrest or to undergo apoptosis (Mellor & Munn 1999) (Figure 1.3). In addition, an accumulation of the metabolites
of the kynurenine pathway such as kynurenine, 3-hydroxykynurenine and 2-hydroxyanthranilic acid can also inhibit directly the proliferation of T-lymphocytes (Fallarino et al. 2002, Frumento et al. 2002). A salient example of the effects of IDO-mediated local immunosuppression is found in the works of Munn and co-workers, who demonstrated that the high level of IDO expression in the placenta plays a key role in suppressing maternal rejection of the fetus by limiting L-Trp availability in the vicinity of the placenta, thereby permitting the allogeneic fetal cells to proliferate in spite of the foreign (paternal) antigens (Munn et al. 1998, Mellor & Munn 2001).

Similarly, the immunosuppressive functions of IDO can be exploited by tumor cells to escape immune detection. IDO expression has been identified in a subset of dendritic cells in tumor draining lymph nodes (TDLNs) (Munn et al. 2004). It is thought that IDO-expressing dendritic cells facilitate immunosuppression by (a) inducing cell-death and anergy in neighboring T-lymphocytes through L-Trp depletion and/or kynurenine metabolites (Fallarino et al. 2002, Frumento et al. 2002, Moffett & Namboodiri 2003, Adams et al. 2004, Munn et al. 2005) and (b) by promoting their differentiation into regulatory T-lymphocytes (T\textsubscript{regs}) of the suppressive phenotype (Fallarino et al. 2006, Curti et al. 2007, Sharma et al. 2007, Sharma et al. 2009) (Figure 1.3). Moreover, T\textsubscript{regs} can also induce IDO expression in dendritic cells by means of a separate signaling pathway in what appears to be a positive feedback loop for immunosuppression (Prendergast 2008, Prendergast et al. 2010). In addition to TLDNs, various human malignant tumors also express IDO. Induction of IDO activity in tumor cells by interferon-\( \gamma \) secreted during inflammatory response alters the tumor microenvironment to be suppressive towards T-lymphocytes proliferation (Uyttenhove et al. 2003), presumably by similar regulatory mechanisms as described in TLDNs. The notion that IDO activity is prevalent in tumors is reinforced by the observation that the level of IDO expression can be an indicator of cancer prognosis (Okamoto et al. 2005, Brandacher et al. 2006).
Figure 1.3 IDO in immunosuppression. In tumor draining lymph nodes and tumors, up-regulation of IDO activity in cancer cells and subset of dendritic cells by interferon-\( \gamma \) depletes the local environment of Trp and increases the concentrations of kynurenine metabolites leading to cell death and anergy of neighboring T-lymphocytes. Alternatively, up-regulation of IDO in dendritic cells indirectly suppresses effector T-lymphocytes via recruitment of T-regulatory lymphocytes (T-reg), which in turn creates a positive feedback loop that stimulates IDO expression by means of a separate signaling pathway.

While there are other mechanisms aside from IDO expression by which tumors escape the host immune response (Pawelec 2004), IDO is of particular interest because it may be amenable to therapeutic intervention. A number of studies based on murine models have demonstrated the use of a small molecule inhibitor of IDO such as 1-methyl-tryptophan (1MT) as a potential therapeutic agent against the progression of tumors (Muller et al. 2005). Friberg and co-workers found that in vivo administration of 1MT delayed the growth of lung carcinoma cells transplanted into syngeneic mice (Friberg et al. 2002). Muller and co-workers demonstrated that established chemotherapeutic agents that are ineffective in treating a mouse model of breast cancer become effective when administered concurrently with 1MT (Muller et al. 2005). Sharma
and co-workers reported that 1MT inhibition of IDO activity in murine dendritic cells reprograms T_{regs} into a non-suppressive, pro-inflammatory phenotype that can stimulate an immune response against tumor cells (Sharma et al. 2009). Interestingly, recent studies revealed that IDO-expressing cells are themselves recognized and targeted by IDO-specific T-lymphocytes (Sorensen et al. 2009, Sorensen et al. 2011) and that circulation of these cells in the host does not appear to induce autoimmunity (Andersen 2012). Consequently, the induction of IDO-specific immune response by means of an IDO-based vaccination has been suggested as an alternative to use of small-molecule inhibitors (Andersen 2012).

1.4 Structure of IDO

The crystal structure of recombinant human IDO in the ferric state was elucidated by Sugimoto and co-workers (Sugimoto et al. 2006). The tertiary structure of IDO is composed of a large domain and a small domain connected by a long loop region (residues 250 to 267; see Chapter 2 for sequence) (Figure 1.4 A). The large domain is comprised of 13 \( \alpha \)-helices (G–S) and two \( 3_{10} \) helices while the small domain consists of six \( \alpha \)-helices (A–F), three \( 3_{10} \) helices, and two short \( \beta \)-sheets. The heme B macrocycle (Figure 1.4 B) binds in a pocket that is delimited on the proximal side by helices G, I, Q and S of the large domain which run parallel to the plane of the heme. The heme iron is coordinated to the protein through a single, proximal histidine (His346) located in helix Q. The distal side of this pocket is mainly covered by the interconnecting loop and helices N, K, and L which interface with the small domain. The structures of two inhibited forms of IDO were included in the same report, one with 4-phenylimidazole (PIM) coordinated to the heme iron, and the other with cyanide (CN\(^{-}\)) coordinated. The heme is bound to IDO such that the 6- and 7-propionates point away and towards the entrance to the active site, respectively, while the 2- and 4-vinyl groups are located.
Figure 1.4 Structure of human IDO in complex with 4-phenylimidazole. (A) The large and small domains are coloured green and blue, respectively. The connecting helices are coloured in cyan. The connecting loop is coloured in red. Heme B and 4-phenylimidazole are coloured yellow and white, respectively. This figure is prepared using the coordinate reported by (Sugimoto et al. 2006) (B) Chemical structure of heme B (iron protoporphyrin IX).
at the back of the heme binding pocket. The 6- and 7-propionates are in *trans* conformation with respect to the plane of the heme, and in *cis* conformation to the heme with respect to their Cα–Cβ bond. The 7-propionate is partially solvent accessible and bends towards the protein to interact with the side-chain of Ser263. Although no L-Trp was observed in the crystal even at high concentrations of substrate (2 mM), clear densities were observed for two molecules of 2-(N-cyclohexylamino) ethane sulfonic acid (CHES, a component of the crystallization buffer) in the distal pocket adjacent to PIM; the authors suggested that L-Trp binds to this region of the active site. It is also possible that the L-Trp binds at the position occupied by PIM instead CHES, although additional L-Trp binding in the region of the ordered CHES molecules may be relevant to the substrate inhibition kinetics that are characteristic of IDO (see section 1.5.1).

1.4.1 Heme environment as revealed by crystallography

The proximal wall of the heme pocket is composed predominantly of hydrophobic residues from the large domain. These residues include Phe214, Ile217, Phe270, Ile329, Val350, Ile354, Leu388, and Phe387. Further scrutiny of the crystal structure reveals a hydrogen-bonding network that connects the proximal histidine (His346) to the 6-propionate by two bridging water-molecules (Wa1 and Wa2; **Figure 1.5**). His346 is hydrogen bonded to Wa1, which interacts with Wa2. Additionally, Wa2 forms a hydrogen bond with the carbonyl of Leu388. The 6-propionate is in contact with Wa2 and is held in position by Arg343, which in turn forms a salt-bridge with Asp274. Notably, replacement of Asp274 with alanine compromises heme binding and abolishes enzymatic activity (Littlejohn *et al.* 2003), suggesting that this hydrogen bond network is structurally and functionally important. Moreover, the His346–Fe bond strength measured by resonance Raman spectroscopy (His–Fe stretching mode = 236 cm⁻¹, (Terentis *et al.* 2002)), is reportedly closer to that found in peroxidases rather than that characteristic of globins,
Figure 1.5 Active-site of human IDO. IDO in complex with 4-phenylimidazole (PIM, purple) and N-cyclohexyl-2-aminoethanesulfonic acid (CHES, yellow).
indicating that His346 is partially anionic (i.e., imidazolate-like) presumably as the result of proton donation to the proximal hydrogen bond network.

The distal side of the heme pocket is comprised of residues from the small and large domains and the interconnecting loop. Because both O₂ and L-Trp bind on this side of the heme, the structural elements of the distal pocket contribute significantly to dioxygenase chemistry. Specifically, the L-Trp binding site appears to be formed by aromatic residues Tyr126, Phe163, and Phe226 (Figure 1.5). By analogy to PIM, L-Trp binding may be mediated through π-π stacking interactions of the indole ring with Tyr126 and Phe163 so that the indole ring is wedged between these two aromatic side chains and held normal to the plane of the heme. Moreover, Arg231 located at the periphery of the heme pocket may interact with the carboxylate group of L-Trp to point the pyrrole ring downwards towards the heme iron and to facilitate reactivity at this side of the indole ring. Other polar residues close to the heme group include Ser167, and Ser263, but neither of these residues appears to be necessary for catalysis (Sugimoto et al. 2006, Chauhan et al. 2008). Interestingly, Ser263 is located at the hinge region of the interconnecting loop (residues 260–265; Ala-Gly-Gly-Ser-Ala-Gly) and it is in contact with the 7-propionate. Comparison of the PIM-bound and CN⁻-bound structures indicates that CN⁻ binding results in substantial displacement of this hinge region that may resemble conformational changes that could be induced upon substrate/ligand binding (Sugimoto et al. 2006). The observation that CN⁻-bound enzyme (IDOFe³⁺–CN⁻) exhibits ~50-fold greater affinity for L-Trp than does IDOFep³⁺ (Kₐ 18 ± 3 µM versus 9.0 ± 0.9 mM, (Lu et al. 2010)) is at least consistent with movement of this hinge region upon substrate binding.

1.4.2 Spectroscopic characterization of the heme environment

IDO can be isolated in various oxidation and ligation states and in substrate-free or substrate-bound forms. Many of these forms have been characterized extensively through
electron paramagnetic resonance spectroscopy (EPR), and magnetic circular dichroism spectroscopy (MCD) (Shimizu et al. 1978, Uchida et al. 1983, Sono & Dawson 1984, Terentis et al. 2002, Lewis-Ballester et al. 2009, Davydov et al. 2010, Yanagisawa et al. 2010, Yanagisawa et al. 2011). These spectroscopic studies provide valuable insights into the heme iron coordination environment for the catalytically relevant forms of IDO (i.e., the substrate-free, O$_2$-bound, and L-Trp bound forms), which have thus far eluded crystallographic characterization. The following sections review this spectroscopic literature and the current state of our understanding of IDO chemical properties. First, however, it is useful to consider briefly the physical basis of the heme iron electronic absorption spectrum and consider the basic electronic properties of the heme iron centre to help in describing the spectroscopic results reported in these studies and in subsequent chapters of this dissertation.

1.4.2.a Electronic absorption spectroscopy

When a molecule absorbs electromagnetic radiation, an electron is promoted from the ground state to an excited state if the absorbed energy is exactly equal to the energy difference ($\Delta E$) between the two states. Generally, this electronic transition occurs from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) (Pavia et al. 2001). However, not all possible electronic transitions (i.e., with appropriate $\Delta E$) are observed, as these transitions are often opposed by physical considerations as specified by selection rules. The probability that a photon of the appropriate energy $\Delta E$ is absorbed by the molecule to promote an electron to an excited state is related to the transition moment integral,

$$\int \psi_e \hat{M} \psi_g d\tau$$

where $\psi_e$ and $\psi_g$ are the total wave functions for the excited and ground state, respectively, and $\hat{M}$ is the electric dipole moment operator which couples these two states (Solomon & Bell 2010).
The selection rules for electronic transitions define the conditions for which the transition moment integral is non-zero. When it is zero, the electronic transition is said to be forbidden (i.e., no probability of occurring and, thus, no absorption intensity). Because $\psi_e$, $\psi_g$, and $\hat{M}$ are related through symmetry, symmetry arguments and group theory can be used to predict whether the direct product of these quantities is zero or non-zero but the magnitude of the integral, which is related to the absorption intensity, should be obtained from experiment or theory.

Experimentally, electronic transitions are coupled at room temperature with a vast number of vibrational transitions resulting in a broad band in the energy spectrum with roughly Gaussian distribution. This convolution reflects the influence of molecular vibrations that are excited in the course of electronic transitions, and it is commonly rationalized as first described by the Franck-Condon principle (Condon 1926). Molecular vibrations also perturb the symmetry of the molecule and so relax the constraints of the symmetry-derived selection rules. As a result, pure electronic transitions that are formally forbidden by these constraints become weakly-allowed through vibronic transitions, and may be observed. The intensities of absorption bands arising from these transitions are correspondingly weaker than those arising from fully-allowed transitions (McMillin 2010).

1.4.2.b Physical basis of the heme electronic absorption spectra

In general, the electronic absorption spectrum of heme (iron protoporphyrin IX; Figure 1.6 A) exhibits an intense absorption maximum at 400–430 nm that is known as the Soret, $\gamma$- or B-band (Platt 1956). Two visible bands often appear in the range 500–600 nm and these are referred to as Q-bands (Platt 1956). Alternatively, these Q-bands are referred to as $\alpha$- and $\beta$-bands, in the order of increasing energy. In some cases, a relatively weak absorption band is also observed at wavelengths $>600$ nm, and this band has been assigned as a charge transfer (CT) transition (Williams 1956).
Figure 1.6 Electronic absorption spectra of IDO. (A) Spectra of the dioxygen adduct of IDO (solid line) and the native IDO in the ferric oxidation state (dashed line). (B) Left: diagrams of the four frontier orbitals ($a_{1u}$, $a_{2u}$, $e_{gx}$, $e_{gy}$) and the four electronic transitions ($a_{1u} \rightarrow e_{gx}$, $a_{1u} \rightarrow e_{gy}$, $a_{2u} \rightarrow e_{gx}$, $a_{2u} \rightarrow e_{gy}$) used to describe the electronic absorption spectrum of porphyrins (Gouterman 1961). Right: relative energy levels of the ground state ($^1A_{1g}$) and excited states ($^1E_u$). The dashed lines correspond to the energies the $^1E_u$ states would have in the absence of electron-electron interactions, whereas the thick, solid lines correspond to the energies of the observed electronic states due to configuration interaction. The thin lines represent different vibrational states of the excited states. Soret and Q$_0$ band arise from pure electronic excitation, and Q$_v$ includes one mode of vibrational excitation (adapted from (McMillin 2010)).
The earliest theoretical model that accounts for the electronic transitions in free base porphyrin was proposed by Simpson (Simpson 1949). In Simpson’s free-electron model, the π-electrons of the porphyrin macrocycle are assumed to be confined to a circular path formed by the inner 18 membered conjugated ring. In the ground state, the 18 π-electrons fill orbitals of magnetic quantum number $M_L = 0, \pm 1, \pm 2, \pm 3, \pm 4$, with $M_L = \pm 4$ and $\pm 5$ corresponding to HOMO and LUMO, respectively. In this model, the lowest-energy excited states arise from four transitions, two of which have orbital angular momentum change $\Delta L$ of $\pm 1$ and are fully allowed. The other two transitions have $\Delta L$ of $\pm 9$ and are forbidden by selection rules. Further, according to Hund’s rule (i.e., for a given multiplicity, the term with the largest value of the orbital angular momentum has the lowest energy), the excited singlet states with $\Delta L$ of $\pm 1$ should have higher energy than those with $\Delta L$ of $\pm 9$. Therefore, this model identifies the intense Soret absorption band as resulting from the former pair of allowed transitions, and the lower energy transitions that give rise to the Q absorption bands as resulting from the latter pair of forbidden transitions, which only gain intensity through coupling to vibrational transitions.

The four-orbital model of Gouterman is an extension of the free electron model that successfully explains the energy gap between the Soret and Q-bands of metallo-porphyrins (Gouterman 1961). In this model, which assumes that the porphyrin has $D_{4h}$ symmetry, Hückel calculations indicate that the HOMOs are a pair of degenerate π-orbitals of $a_{1u}$ and $a_{2u}$ symmetry and the LUMOs are a pair of degenerate π-antibonding-orbitals of $e_{gx}$ and $e_{gy}$ symmetry (Figure 1.6 B). The electron configurations of the excited states are $(a_{1u})^2(a_{2u})^1(e_{gx})^1$, and $(a_{1u})^2(a_{2u})^1(e_{gy})^1$ for the Q-bands, and $(a_{1u})^1(a_{2u})^2(e_{gy})^1$ and $(a_{1u})^1(a_{2u})^2(e_{gx})^1$ for the Soret band. Because the Soret and Q-bands excited states are both $^1E_u$ in character (i.e., singlet excited state with $e_u$ symmetry), they mix together, and the system is said to undergo configuration interaction. As a result, the degeneracy of the transition energies for the Soret and Q-bands excited states is lifted and causes
the Soret band to appear at a much higher energy than the Q-bands. Also, when the \(a_{1u}\) and \(a_{2u}\) orbitals are approximately equal in energy, orbital mixing results in much smaller transition moments for the Q-bands than for the Soret band (Fukuda & Kobayashi 2010), which is consistent with the greater absorption intensity of the Soret band. In cases where the degeneracy of the \(a_{1u}\) and \(a_{2u}\) orbitals is lifted (e.g., by interaction with the orbitals of the central iron), the intensity of the Q-bands increases at the expense of the Soret band, and the separation between the two bands decreases.

Major factors that affect the electronic absorption spectrum of iron porphyrin include the oxidation and spin states of the central iron atom. Iron is usually present in the ferric (\(\text{Fe}^{3+}; [\text{Ar}]3d^54s^0\)) or ferrous (\(\text{Fe}^{2+}; [\text{Ar}]3d^64s^0\)) state. The five degenerate \(d\)-orbitals of iron split under the influence of various ligand field geometries (although tetragonal and octahedral coordination geometries are the most relevant to iron porphyrins). The splitting pattern is dependent on the coordination number and nature of the ligand and dictates the distribution of electrons within the \(d\)-orbitals (Moore & Pettigrew 1990). In a 6-coordinate octahedral system where the ferric iron has one axial histidine ligand and one axial water ligand, the preferred electronic configuration of the five \(d\)-electrons is generally \((d_{xy})^1(d_{xz})^1(d_{yz})^1(d_{x^2-y^2})^1\), commonly referred to as high-spin (i.e. \(S=5/2\)). Replacement of water by a strong field ligand such as cyanide changes the \(d\)-orbital splitting pattern such that the low-spin (i.e. \(S=1/2\)) configuration \((d_{xy})^2(d_{xz})^2(d_{yz})^1\) is favoured. The \(d-d\) transitions of iron are generally (electric-dipole) forbidden (McMillin 2010), so the contributions of these transitions to the electronic spectrum are obscured by the \(\pi-\pi^*\) transitions of the porphyrin.

Depending on the energy levels and occupancies of the iron \(d\)-orbitals relative to those of the porphyrin \(\pi\)-orbitals, electronic transitions from porphyrin \(\pi\)-orbitals to iron \(d\)-orbitals are possible and lead to the appearance of charge transfer (CT) absorption bands (Smith & Williams 1970, Gouterman 1978, Makinen & Chung 1983). In the case of ferric IDO in the high-spin
configuration, a CT band is observed at 630 nm that is absent from the spectra of the ferrous high spin or ferric low-spin protein. In addition, depending on the type of ligand, CT bands can also arise from electronic transitions from the axial ligand $\pi$-orbitals to iron $d$-orbitals (Smith & Williams 1970, Makinen & Chung 1983). Finally, the CT and $d$-$d$ transitions can mix and shift the porphyrin $\pi$-$\pi^*$ transitions (Adar 1978). Consequently, the heme electronic absorption spectrum is highly sensitive to the electronic configuration of the central iron atom. As a result, it is possible to obtain information on the oxidation state, spin state, and to some extent, the identity of the axial ligands in heme proteins through conventional electronic absorption spectroscopy although a variety of spectroscopic techniques are generally required to characterize the active-sites of heme proteins adequately.

1.4.2.c Heme coordination environment of substrate-free IDO

Prior to the elucidation of the crystal structure of IDO, spectroscopic studies of the enzyme were concerned primarily with the identification of the heme iron coordination environment. In general, the spectroscopic properties of IDO in various oxidation and ligation states are very similar to those of myoglobin (Uchida et al. 1983, Sono & Dawson 1984). The heme Fe$^{2+}$ and Fe$^{3+}$ of substrate-free IDO are 5- and 6-coordinate, respectively. The porphyrin pyrrole ring nitrogen atoms are the equatorial ligands to the central iron atom, and the proximal axial ligand has been identified as histidine (His346) (Shimizu et al. 1978, Uchida et al. 1983, Sono & Dawson 1984, Littlejohn et al. 2003), but there has been conflicting evidence as to the identity of the sixth axial ligand in the ferric enzyme. The electronic absorption, EPR and MCD spectra of rabbit IDO all indicate that the heme Fe$^{3+}$ exists as a mixture of high-spin ($S$=5/2) and low-spin states ($S$=1/2) with the high-spin form dominating at room temperature and neutral pH (Uchida et al. 1983, Sono & Dawson 1984). This spin-state equilibrium is temperature dependent but not pH dependent, suggesting that the low-spin state does not result from an acid-base
transition as seen in myoglobin. As a result, the mixed-spin character of ferric IDO (IDOFe\(^{3+}\)) has been attributed to a sterically-hindered distal histidine (His/His axially coordinated, (Uchida et al. 1983, Sono & Dawson 1984)), and similar conclusions resulted from subsequent spectroscopic studies of the human enzyme (Papadopoulou et al. 2005). On the other hand, Terentis and co-workers observed a minor increase in low-spin character of human IDOFe\(^{3+}\) at alkaline pH by resonance Raman spectroscopy (Terentis et al. 2002). Based on the similarity of the resonance Raman spectra of IDOFe\(^{3+}\) to those of ferric myoglobin, these authors suggest that the sixth ligand of IDOFe\(^{3+}\) is a weak-field H\(_2\)O ligand at neutral pH and strong-field hydroxide anion (OH\(^-\)) at alkaline pH. Although the spectroscopic origin of low-spin IDOFe\(^{3+}\) requires further confirmation, it is clear from the crystal structure that no histidine residues (other than His346) are near the heme iron, which renders His/His coordination possible only as the result of a drastic change in the protein fold.

1.4.2.d Heme coordination environment of L-Trp-bound IDO

Binding of L-Trp to IDOFe\(^{3+}\) shifts the predominantly high-spin heme iron to the low-spin state, particularly at alkaline pH. The origin of the L-Trp induced spin-state change appears to have been definitively assigned as the His/OH\(^-\) axial coordination of heme Fe\(^{3+}\) (Sono & Dawson 1984, Terentis et al. 2002). In the presence of L-Trp, the vibrational bands at 546 and 496 cm\(^{-1}\) in the resonance Raman spectrum of IDOFe\(^{3+}\) have been assigned to Fe–OH\(^-\) stretching modes by Terentis and co-workers on the basis of their similarity to the alkaline forms of myoglobins (Terentis et al. 2002). As these bands are very weak for IDOFe\(^{3+}\) in the absence of L-Trp even at alkaline pH, these authors further suggest that the heme bound OH\(^-\) is stabilized through hydrogen bonding to the indole nitrogen proton of L-Trp.

IDO activity has long been known to be inhibited at high concentrations of L-Trp (i.e., substrate inhibition), implying the presence of a second L-Trp binding site. Sono reported that
titration of a homogeneous preparation of L-Trp-bound IDOFe$^{3+}$ (IDOFe$^{3+}$–L-Trp) by non-substrates indole and indoleethanol leads to further perturbation of the MCD and electronic absorption spectra of the IDOFe$^{3+}$–L-Trp complex (Sono 1989). Because these non-substrates modulate IDO activity, the author suggests the possibility of an allosteric site in IDO that could bind effectors or a second molecule of L-Trp. Lu and co-workers later corroborated the two-binding sites model by demonstrating binding of two equivalents of L-Trp to the IDOFe$^{3+}$–CN$^-$ complex (Lu et al. 2010).

Unlike IDOFe$^{3+}$, binding of L-Trp to ferrous IDO (IDOFe$^{2+}$) does not alter the coordination environment of the heme iron (i.e., 5-coordinate, $S$=2). Consequently, both IDOFe$^{2+}$ and L-Trp-bound IDOFe$^{2+}$ (IDOFe$^{2+}$–L-Trp) can bind O$_2$ at the sixth coordination site (second order rate constants of O$_2$ binding are 7.4 × $10^6$ and 6.3 × $10^6$ M$^{-1}$ s$^{-1}$ for IDOFe$^{2+}$ and IDOFe$^{2+}$–L-Trp, respectively, (Taniguchi et al. 1979)). Moreover, the nature of the proximal His346–Fe$^{2+}$ bond appears to be unchanged so that it retains a peroxidase-like character upon binding of L-Trp, as indicated by unchanged His–Fe stretching modes (236 cm$^{-1}$) regardless of whether L-Trp is present or not (Terentis et al. 2002). IDOFe$^{2+}$ also exhibits significantly greater affinity for L-Trp than does IDOFe$^{3+}$ (dissociation constants ($K_d$) 13 µM and 5.8 mM, respectively at pH 7.0 for the rabbit enzyme, (Sono et al. 1980)).

1.4.2.e L-Trp–O$_2$ interactions of the ternary complex

The valence structure of IDO with O$_2$ bound can be represented as either ferrous-oxy, (IDOFe$^{2+}$–O$_2$), or ferric-superoxide (IDOFe$^{3+}$–O$_2^•$); the latter form is generally a more accurate description of heme iron bound O$_2$ (Wittenberg et al. 1970) and has been corroborated by recent resonance Raman studies (O–O stretching mode for IDOFe$^{3+}$–O$_2^•$ = 1137 cm$^{-1}$, (Lewis-Ballester et al. 2009); metal–O$_2^•$ = 1100-1150 cm$^{-1}$; O$_2$ gas = 1556 cm$^{-1}$, (Das et al. 2001)).

With the exception of some mechanistic proposals discussed in section 1.5.2, the IDOFe$^{3+}$–O$_2^•$
representation of the oxygenated enzyme is assumed throughout this dissertation. The ternary complex of heme iron, O₂, and substrate (IDOFe³⁺−O₂⁻−L-Trp) is short-lived due to the rapid turnover to produce N-FK (Hirata et al. 1977, Taniguchi et al. 1979), although this complex can be stabilized at −30°C (Sono 1986). Consequently, spectroscopic studies of the IDO ternary complexes generally substitute O₂ with inert ligands such as cyanide or carbon monoxide (i.e., IDOFe³⁺−CN⁻ and IDOFe²⁺−CO, respectively). For example, using MCD spectroscopy to study the effects of L-Trp binding to rabbit IDOFe³⁺−CN⁻ and IDOFe²⁺−CO, Uchida and co-workers observed a decrease in the Soret band intensity, which they attribute to a shift from linear conformations of the Fe−C−O or Fe−C−N bonds to bent conformations due to steric constraints imposed by the bound L-Trp (Uchida et al. 1983). These findings have been corroborated with subsequent resonance Raman studies of the human enzyme (Terentis et al. 2002).

Davydov and co-workers applied cryoreduction, conventional EPR and pulsed EPR (¹H-ENDOR) techniques to the characterization of the IDOFe³⁺−O₂⁻−L-Trp ternary complex (Davydov et al. 2010) in studies that led to the suggestion that binding of L-Trp disrupts hydrogen bonds between O₂ and active site water molecules that otherwise stabilize the Fe³⁺−O₂⁻⁻ bond. Also, based on the similarities in the EPR and ¹H-ENDOR spectra of the ternary complexes involving either L-Trp or 1MT (which lacks the proton of indole nitrogen), the authors further propose that L-Trp and 1MT stabilize heme-bound O₂ by hydrogen bonding to the side-chain amino group and that this interaction may be crucial to dioxygenase activity.

1.5 Enzymology of IDO

IDOFe³⁺ also reacts readily with superoxide (O₂⁻⁻) to yield IDOFe³⁺−O₂⁻⁻, which is formed by the binding of O₂ to IDOFe²⁺ as noted above. The second order rate constant for the reaction of (rabbit) IDOFe³⁺ with O₂⁻⁻ is similar to that for reaction of IDOFe²⁺ with O₂ (7 × 10⁶ M⁻¹ s⁻¹ and 6.3–7.4 × 10⁶ M⁻¹ s⁻¹, respectively, (Taniguchi et al. 1979, Kobayashi et al. 1989))
and substantially greater than those of the corresponding reactions of ferric TDO, ferric myoglobin, and ferric cytochrome P450, all of which display relatively low reactivity towards \( \text{O}_2^{\cdot-} \) (Ilan et al. 1976, Sutton et al. 1976, Debey et al. 1979).

Considering the reducing environment of the cytosol, the majority of IDO is likely to occur in the active, \( \text{Fe}^{2+} \) state under physiological steady-state conditions. However, IDO is generally purified in the inactive \( \text{Fe}^{3+} \) state, so activation \textit{in vitro} requires initial reduction of the enzyme. The approach most widely used in steady-state activity measurements of the enzyme relies on a methylene blue/ascorbate coupled system (Yamamoto & Hayaishi 1967, Shimizu et al. 1978, Sono 1989, Sono & Cady 1989, Littlejohn et al. 2003, Poljak et al. 2006, Carr et al. 2008), in which according to Sono, the dye acts primarily as mediator to facilitate electron transfer from ascorbic acid to the enzyme (Sono 1989). \( \text{O}_2^{\cdot-} \) generated as byproduct of the reaction of ascorbate with \( \text{O}_2 \), apparently, is not a significant reductant provided sufficient methylene blue is present (Sono 1989). On the other hand, a mixture of xanthine oxidase and hypoxanthine activates IDO strictly by formation of \( \text{O}_2^{\cdot-} \) (Sono 1989). With increasing concentrations of xanthine oxidase and hypoxanthine, IDO activity supported by these agents can reach the apparent maximal level that can be achieved by methylene and ascorbic acid (Sono 1989).

Although \( \text{O}_2^{\cdot-} \) can be a highly effective reductant of IDO \textit{in vitro}, it is less likely to be the major physiological, \textit{in vivo} reductant owing to the ubiquitous presence of superoxide dismutase. Instead, cytochrome \( b_5 \), cytochrome \( b_5 \) reductase, and \( \beta \)-NADH have been shown to form part of an important mechanism responsible for physiological reduction of IDO (Vottero et al. 2006, Maghzal et al. 2008), in a manner analogous to the reduction of hemoglobin in red blood cells. Moreover, as discovered during the current study and discussed in later chapters, \( \beta \)-NADH alone is sufficient to activate IDO \textit{in vitro}. 
1.5.1 Reaction cycle

The minimal catalytic cycle of IDO is shown in Figure 1.7 A. The ternary complex IDOFe$^{3+}$–O$_2^*$–L-Trp forms upon binding of O$_2$ to IDOFe$^{2+}$ followed by L-Trp (Hirata et al. 1977, Sono et al. 1980). This order of binding is in contrast to other heme-containing oxygenases such as TDO and cytochromes P450 that require substrate to bind prior to O$_2$ (Ishimura et al. 1970, Ishimura et al. 1971, Peterson et al. 1972, Tyson et al. 1972). Binding of O$_2^*$ to IDOFe$^{3+}$ also presumably precedes L-Trp binding because L-Trp inhibits reaction of O$_2^*$ with IDOFe$^{3+}$ (Kobayashi et al. 1989). At higher L-Trp concentrations, the order of binding reverses. Because the substrate inhibition constants track the dissociation constants for L-Trp binding to IDOFe$^{3+}$ closely as a function of pH, Sono and co-workers suggest that the IDOFe$^{3+}$–L-Trp complex contributes to substrate inhibition of IDO (Sono et al. 1980).

Once formed, the IDOFe$^{3+}$–O$_2^*$–L-Trp complex transfers two atoms of oxygen to L-Trp to yield N-FK. This transfer may proceed in a concerted or sequential fashion, and mechanistic speculations concerning this step are discussed in detail in sections 1.5.2. Importantly, the process of oxygen transfer regenerates IDOFe$^{2+}$. So in principle, once IDOFe$^{2+}$ is formed, oxidation of L-Trp by IDO does not require additional reducing equivalents. However, IDOFe$^{3+}$–O$_2^*$ auto-oxidizes to IDOFe$^{3+}$ with the release of O$_2^*$, both in the presence and absence of L-Trp (first order rate constants are $2.8 \times 10^{-2}$ and $4.7 \times 10^{-4}$ s$^{-1}$ respectively, for the rabbit enzyme, (Hirata et al. 1977, Taniguchi et al. 1979)). Consequently, reduction of the enzyme is required again to re-activate catalysis.

For comparison to the dioxygenase reaction cycle of IDO, the monooxygenase reaction cycle of cytochromes P450 is shown in Figure 1.7 B (Sono et al. 1996). As noted earlier,
Figure 1.7 Catalytic cycle of IDO in comparison to cytochromes P450. The porphyrin macrocycle is omitted for clarity. (A) Catalytic cycle of IDO. \(\lambda\)-Trp turnover in isolation does not require input of electrons aside from that which is needed to reactivate enzymes that have auto-oxidized back to \(\text{IDOFe}^{3+}\) via route \(a\). Binding of \(O_2\) precedes binding of \(\lambda\)-Trp. At high concentrations of \(\lambda\)-Trp, \(\lambda\)-Trp binding to \(\text{IDOFe}^{3+}\) appears to be a cause of substrate inhibition. (B) Catalytic cycle of cytochromes P450. The conversion of substrate \(RH\) to \(ROH\) by insertion of one atom of oxygen consumes two electrons and two \(H^+\) per turnover, which can be supplied in the form of hydrogen or organic peroxides (route \(b\)).

substrate binding to cytochromes P450 precedes \(O_2\) binding. In fact, it is only after an accompanying change in the heme \(Fe^{3+}\) from low-spin to high-spin state and a one electron reduction of the heme \(Fe^{3+}\) to \(Fe^{2+}\) that binding of \(O_2\) can occur to form the ternary complex. The
monooxygenase and dioxygenase reaction pathways of the two enzymes diverge after formation of this complex. In the case of cytochrome P450, addition of a second electron to the ternary complex yields the ferric peroxo ($\text{Fe}^{3+}-\text{OO}^-$) adduct, which is subsequently protonated to yield the ferric-hydroperoxide ($\text{Fe}^{3+}-\text{OOH}$) species. A second protonation results in heterolytic cleavage of the O–O bond to yield H$_2$O and a ferryl ($\text{Fe}^{4+}=\text{O}$) porphyrin cation radical intermediate commonly known as Compound I. The ferryl oxygen of Compound I is then transferred to the substrate with regeneration of the starting $\text{Fe}^{3+}$ form of the protein. Cytochrome P450 turnover, therefore, consumes two reducing equivalents and two H$^+$ per catalytic cycle. The two electrons and two H$^+$ can be supplied in the form of hydrogen peroxide (H$_2$O$_2$), alkyl hydroperoxides, peracids, and sodium chlorite (Hrycay et al. 1975, Nordblom et al. 1976). These oxygen donors react with the ferric enzyme to oxidize substrates directly, bypassing the $\text{Fe}^{3+}-\text{O}_2^-$ and $\text{Fe}^{3+}-\text{OO}^-$ enzyme adducts. This reaction shortcut is generally referred to as the peroxide shunt. In stark contrast, IDO is unable to catalyze oxidation of L-Trp by H$_2$O$_2$ (Hayaishi 1976, Ferry et al. 2005, Lu & Yeh 2011), indicating that oxidation of IDO$\text{Fe}^{3+}-\text{O}_2^-$ to more highly oxidized species disfavours L-Trp dioxygenase activity and is probably avoided under typical reaction conditions. Nevertheless, oxygen transfer by IDO can be achieved via a peroxide shunt with substrates other than L-Trp as shown in later chapters.

1.5.2 Proposed mechanisms of L-Trp oxidation

The mechanism by which ternary complexes of IDO and TDO transfer O$_2$ to L-Trp has been the subject of continuing debate since the discovery of these enzymes more than 40 years ago. The elucidation of the crystal structures of IDO and TDO, in combination with density functional theory (DFT) and other quantum mechanics based computational techniques (Chung et al. 2008, Capece et al. 2010, Chung et al. 2010, Capece et al. 2012), have contributed significantly to the identification of new plausible reaction mechanisms although no consensus
mechanism has been identified. These mechanisms may be grouped into two general types: (1) transfer of O₂ involving a single Fe–O bond cleavage, and (2) step-wise transfer initially involving O–O bond cleavage and then Fe–O bond cleavage. The discussion of these mechanisms that follows focuses on IDO even though some of these mechanisms result from studies on TDO. This approach is reasonable because IDO and TDO are believed to catalyze L-Trp oxidation by the same general pathway even though some specific differences in their mechanisms are noted explicitly.

1.5.2.a Transfer of O₂ involving a single Fe–O bond cleavage

The earliest mechanistic proposals for the catalytic mechanism of IDO involve abstraction of the proton of indole nitrogen by an active-site base (Figure 1.8, steps A1, B1). Hamilton suggests that this step is then followed with nucleophilic attack by the electron-rich L-Trp C₃ on the distal oxygen of the coordinated O₂, which is described as IDOFe²⁺–O₂ (step A2, (Hamilton 1969)). The heme substitution studies of Makino and co-workers, which demonstrated increasing L-Trp oxidation rates with stronger electron-withdrawing 2,4-porphyrin substituents, support this mechanism insofar as increased electrophilicity at O₂ should enhance nucleophilic attack by L-Trp C₃ (Makino et al. 1980). In contrast to the ionic pathway of Hamilton, Leeds and co-workers propose a radical pathway involving one electron oxidation of a L-Trp C₃ anion by the heme-bound O₂ (step B2), which in this case is formulated as IDOFe³⁺–O₂⁻ (Leeds et al. 1993). This step is followed by radical recombination of the L-Trp C₃ radical and IDOFe²⁺–O₂⁻ (step B3). However, the feasibility of such an electron transfer reaction has been questioned in view of the high reduction potential of L-Trp (Sono et al. 1996). In both the ionic and radical pathways, an IDOFe²⁺–3-indolenylperoxy complex (I) intermediate is formed.
Figure 1.8 IDO-catalyzed oxidation of L-Trp by abstraction of the indole nitrogen proton. See section 1.5.2 for details.
Hamilton suggests that (I) decomposes by cleavage of the Fe–O bond to yield a metastable dioxetane intermediate (II), although the high energy barrier generally associated with dioxetane formation may make this step (D1) unfavourable (Hamilton 1969). On the other hand, recent DFT calculations by Chung and co-workers suggest that the energy barrier for the dioxetane intermediate is relatively low if the distal oxygen is bonded to C2 of the indole ring (i.e., to form an IDOFe$^{2+}$-2-indolenylperoxy complex), whereas the energy barrier is higher if oxygen adduct formation occurs at C3 (Chung et al. 2008). Nevertheless, subsequent ring opening of a dioxetane intermediate to yield the final product N-FK (step D2) is expected to be highly exothermic; DFT calculations predict this final step to be essentially barrierless (Chung et al. 2008).

Parts of these early proposals, specifically the step concerning base-catalyzed proton abstraction, have been ruled out by more recent structural and mutagenesis studies of human IDO. Ser167 and Ser263 of human IDO, which are the only possible candidates for the active-site base, have been shown to be unimportant for catalysis (Sugimoto et al. 2006, Chauhan et al. 2008). Therefore, it appears that IDO dioxygenase activity does not involve an active-site base, prompting Sugimoto, Terentis and co-workers to advance independently the idea that the heme-bound O2 abstracts the proton of indole nitrogen (Terentis et al. 2002, Sugimoto et al. 2006). These authors proposed that L-Trp binds in a manner that enables hydrogen bonding of the indole nitrogen proton to the proximal oxygen of the heme-bound O2. This interaction facilitates proton abstraction by the proximal oxygen and electrophilic addition of the distal oxygen attacking the C2–C3 $\pi$-bond (step C1) to yield 3-hydroperoxy-indolenine, which subsequently rearranges to form a dioxetane intermediate (step C2).

In the case of human TDO, the experimental evidence supports catalytic involvement of an active-site base. Resonance Raman studies of TDO suggest that in contrast to IDO, the indole nitrogen proton does not interact with the heme-bound O2 (Batabyal & Yeh 2007). These authors
also showed through mutagenesis that His77, which is located in the distal heme pocket of the protein by structural homology to *Xanthomonas campestris* TDO, appears to be crucial for enzymatic activity.

### 1.5.2.b Step-wise transfer of O$_2$ involving ferryl intermediate

More recent mechanistic developments based on quantum and molecular mechanics computations have challenged the long-held belief that L-Trp is activated by deprotonation of the indole nitrogen proton. In a search for plausible transition states, Chung and co-workers propose that abstraction of the indole nitrogen proton, which has a p$K_a$ of $\sim$17 (Yagil 1967), is energetically unfavourable (Chung *et al.* 2008). As well, Chauhan and co-workers demonstrated that 1MT is in fact a substrate for IDO, arguing against an obligate deprotonation step (Chauhan *et al.* 2008). Two alternatives have been suggested (Figure 1.9): (a) direct electrophilic addition (step E1, (Chung *et al.* 2008, Chauhan *et al.* 2009)) or (b) direct radical addition of the distal oxygen of IDOFe$^{3+}$–O$_2^*$ to the C$_2$–C$_3$ π-bond of indole (step F1, (Chung *et al.* 2008, Chauhan *et al.* 2009, Chung *et al.* 2010, Efimov *et al.* 2011)). In the latter case, oxygen insertion at C$_2$ has been calculated to be more favourable on thermodynamic grounds than at C$_3$, so this configuration is depicted (Figure 1.9). Both mechanisms lead to formation of an IDOFe$^{2+}$–2-indolenylperoxy complex in the singlet state or an IDOFe$^{2+}$–2-indolenylperoxy radical complex that subsequently converts to **II** by ionic and radical mechanisms, respectively. Interestingly, simulations also predict that O–O bond cleavage competes energetically with the formation of **II**, leading instead to the formation of a ferryl (IDOFe$^{4+}$=O) intermediate and metastable tryptophan-2,3-epoxide (**III**; step G1, (Capece *et al.* 2010, Chung *et al.* 2010)). Experimental evidence for the involvement of IDOFe$^{4+}$=O during catalytic turnover of L-Trp has been provided by resonance Raman studies (Lewis-Ballester *et al.* 2009, Yanagisawa *et al.* 2010), while formation of **III** has been inferred by Basran and co-workers based on the observation of
Figure 1.9 IDO-catalyzed oxidation of L-Trp by electrophilic or radical addition of oxygen to the indole $\pi$-bond. See section 1.5.2 for details.
3α-hydroxypyrroloindole-2-carboxylic acid as a minor product of the reaction of L-Trp with IDO (Basran et al. 2011). These findings strongly support the conclusion that transfer of O₂ to substrate by IDO and TDO occurs in a sequential manner.

The conversion of III to N-FK has been suggested to be initiated by epoxide ring opening by heterolytic cleavage of the C₂–O bond (step G2, (Chung et al. 2010, Basran et al. 2011, Capece et al. 2012)). In this process, charge stabilization of the incipient oxy anion is in turn mediated by the L-Trp amine group. Proton transfer is also a possibility. This reaction is followed by attack of the ferryl oxygen at the C₂ cation and finally cleavage of the Fe–O bond to yield N-FK (steps G3–G4). In this respect, insertion of the second oxygen atom resembles the corresponding process in the mechanism of monooxygenases.

The extent of mechanistic similarity of IDO and TDO to cytochrome P450 monooxygenases is unclear although Basran and co-workers suggest that it is reasonable that a reactivity pattern should emerge across the family of heme-containing oxygenases that share the same heme structure and similar reactivity with O₂ (Basran et al. 2011). Review of the vast literature concerning the mechanism of oxygen transfer by cytochromes P450 is beyond the scope of the current discussion although it is clear that a one-solution-fits-all mechanism that describes the highly diverse and substrate-dependent reactivities exhibited by this class of enzymes (i.e. alkane hydroxylation, arene epoxidation, N-dealkylation, alcohol oxidation, C–C bond cleavage, etc.) is unlikely. Nevertheless, those reactions relevant to oxygen insertion in C–H bonds and C–C π-bonds are considered further within the context of the new reactions of IDO presented in Chapter 4.
1.6 Thesis objectives

Many of the fundamental catalytic and functional properties of IDO were characterized by elegant studies of Hayaishi and co-workers following their discovery of the enzyme. In subsequent years, this enzyme fell into relative obscurity, perhaps because of the difficulty in purifying homogeneous IDO from the usual biological source (i.e., rabbit intestine) and the lack of a recombinant expression system until 2000 (Littlejohn et al. 2000). Interest in IDO was revitalized following the recognition of its roles in T-lymphocyte suppression, and significant developments concerning the physiology, structure and reaction mechanism of the enzyme have been reported in recent years.

The primary objective of the work presented in this dissertation is to characterize two previously unreported reactions of IDO: (a) reductive activation of IDO by the obligate two electron reductant nicotinamide adenine dinucleotide (β-NADH) in the absence of mediators and (b) H₂O₂-dependent, O₂-independent oxidation of indole, which is known to be non-reactive by the normal catalytic cycle (Shimizu et al. 1978). Another objective is to define the basic enzymology and biochemical properties of a recently discovered human IDO isoform that has been variously referred to in the literature as IDO2 (as in this dissertation), proto IDO, or INDOL1 (Metz et al. 2007, Ball et al. 2009). To avoid confusing the two enzymes, the form of IDO discovered by Hayaishi and co-workers is referred to in this dissertation as IDO1.

Excerpts of this work were published prior to the completion of this dissertation (Rosell et al. 2011, Kuo & Mauk 2012), and others are in preparation.
Chapter 2: materials and methods

2.1 Molecular biology

IDO1 was expressed from a pET28-a-(+) vector (Novagen; Darmstadt Germany) containing a gene encoding human IDO1 that had been amplified from a permanent human cell line (Edgell et al. 1983, Vottero 2007) and that was modified by Dr. Federico Rosell to encode a tobacco etch virus (TEV) protease recognition sequence (Glu-Phe-Leu-Tyr-Phe-Gln-Met) between the thrombin cleavage sequence and the IDO1 initiation codon (Figure 2.1; plasmid His-TEV-IDO1). In addition, the codon for Cys308 was mutated to encode a Ser residue to eliminate formation of the intermolecular disulfide bond apparent in the crystal structure of the protein (Sugimoto et al. 2006). Hydrolysis of the recombinant IDO1 expressed from this construct with TEV protease resulted in a product with three extra residues (Gly-Glu-Phe) at the N-terminus of the protein (Figure 2.2). This protein is referred to in this dissertation as wild-type IDO1 despite these changes in sequence relative to the true wild-type enzyme.

Two transcripts have been reported for the human IDO2 gene (407 and 420 residues; Q6ZQW0 (UniProtKB) and NP_919270 (RefSeq), respectively), which have been identified from different tissues (Metz et al. 2007). The longer transcript, regarded here as wild-type IDO2, encodes an additional 13 residues at the N-terminus of the protein but is otherwise identical to the other splice variant. This longer transcript (420 amino acids) was used as a template for an artificial human IDO2 gene that incorporated only codons with relative usage frequency greater than 5% in E. coli (Henaut & Danchin 1996) and included a TEV protease recognition sequence immediately upstream of the IDO2 initiation codon (Figure 2.2; His-TEV-IDO2). This sequence was synthesized by Blue Heron (Bothell, WA) and it was inserted between the BamHI and HinDIII restriction sites of the plasmid pET28-a-(+). In this case, hydrolysis of the resulting enzyme with TEV protease yields a single additional Gly residue at the N-terminus of wild-type IDO2.
Figure 2.1 Functional maps of the 6,541 bp His-TEV-IDO1 and 6,634 bp His-TEV-IDO2 plasmids. Selected restriction sites in the multiple cloning sites and gene insert are indicated in blue (unique) and red (2 sites). The His-TEV-Δ27IDO2 plasmid is not shown here, but it is identical to the His-TEV-IDO1 plasmid except the IDO1 sequence following the TEV cleavage site was replaced entirely with the Δ27IDO2 sequence. The DNA sequences of the plasmids are provided under Appendix A.
Figure 2.2 Translational maps of the His-TEV-IDO1, His-TEV-IDO2, and His-TEV-\(\Delta 27\)IDO2 constructs. His-Tags were removed from the expressed protein by digestion with TEV protease, with cleavage occurring between the Gln and Gly peptide bond as indicated. Only the initiation sequence is shown for His-TEV-\(\Delta 27\)IDO2; the remaining sequence is identical to His-TEV-IDO2. Residues are colour-coded based on polarity.
Secondary structure analysis of the IDO2 sequence by DisEMBL (Linding et al. 2003) predicted that the N-terminal region of this enzyme lacks ordered secondary structure thereby making expression and handling of this protein more difficult than IDO1. Consequently, a truncated form of the IDO2 gene was amplified without the sequence that encodes the 27-residue N-terminal polypeptide fragment by the polymerase chain reaction with the flanking primers 5’- TTTCAGGGCGAATTCTCTCTCAGGAATCCTATCATATCTCCG, and 5’-GTGGTGTTGTGCTCGAGTGCCGCGCAAGCTT. This product was sub-cloned into a linearized pET28-a(+) vector with the in-Fusion Cloning Kit (Clontech; Mountain View, CA). The site of insertion of the truncated sequence was such that TEV protease digestion of the resulting enzyme (expressed from plasmid His-TEV-Δ27IDO2) includes an N-terminal Gly residue followed by Ser28 of the wild-type IDO2 sequence (Figure 2.2). This variant is referred to in this dissertation as Δ27IDO2. In all cases, the plasmids were validated by sequencing (Genewiz, La Jolla, CA). The complete DNA sequences of His-TEV-IDO2 and His-TEV-Δ27IDO2 are provided in Appendix A.

2.2 Protein expression and purification

2.2.1 Reagents

DNase I, hen egg white lysozyme, Cibacron blue 3GA agarose CL, phenylmethylsulfonyl fluoride (PMSF), and all analytical grade buffer (Trizma base, potassium phosphate, sodium phosphate) were from Sigma-Aldrich (St. Louis, MO). Imidazole was obtained from various sources (Fisher, Baker, and Sigma) and was recrystallized from toluene after treatment with activated charcoal to remove impurities that absorb at 280 nm. δ-aminolevulinic acid (ALA) (Chem-Impex International Inc., Wood Dale, IL), kanamycin sulfate (BioBasic Inc., Markham, ON), and IPTG (MolecuLA, Columbia, MD) were obtained from the vendors indicated. TEV protease (plasmid kindly provided by Prof. David S. Waugh, (Lucast et al. 2001)) and bovine
liver microsomal tryptic cytochrome \( b_5 \) were prepared as reported previously (Funk et al. 1990). Human cytochrome \( b_5 \) reductase (plasmid kindly provided by Prof. Lauren Trepanier was prepared by Dr. Tomoko Misono as reported (Kurian et al. 2004). All solutions were prepared with glass distilled water that had been purified further by passage through a Barnstead Nanopure Diamond purification system.

2.2.2 Recombinant human IDO1

Cultures of \textit{E. coli} Rosetta(DE3)pLysS carrying plasmid His-TEV-IDO1 were grown in LB broth (1.6 L per 2 L flask; 50 mg kanamycin/L) at 37 °C and shaken at 250 rpm until the culture reached an \( \text{OD}_{600} \) of 0.8 to 1. The temperature was then lowered to 30 °C, and filter-sterilized \( \delta \)-ALA was added to a final concentration of 0.5 mM. After 30 minutes, filter-sterilized IPTG was added to a concentration of 0.5 mM to induce IDO1 expression. The culture was then grown overnight (150 rpm) before harvesting the cells by centrifugation (6,000g for 15 minutes at 4 °C). Pelleted cells were resuspended in ice-cold sodium phosphate buffer (50 mL, 50 mM, pH 7.5, 0.5 M NaCl), and PMSF dissolved in ethanol was added to achieve a final concentration of 1 mM. This buffer (without PMSF) was used in all subsequent purification procedures unless otherwise specified. DNase I and lysozyme (0.5 and 5 mg, respectively) were added to the cell suspension. Cell lysis was achieved by passing the lysate through an Emulsi-FlexC5 (Avestin, Inc., Ottawa, ON) cell disruptor (three cycles at 17,000 psi).

Following removal of the cell debris by centrifugation (1 hour, 13,000 rpm, Sorvall SS34 rotor, 4 °C), imidazole was added to the cell-free extract to a final concentration of 20 mM. The resulting solution was applied to a \( \text{Ni}^{2+} \)-loaded HiTrap-Chelating HP column (5 mL; GE Healthcare) equilibrated in phosphate buffer with imidazole (20 mM). The column was washed with the same buffer (~200 mL) until the absorbance of the eluent at 280 nm approached zero before eluting IDO1 with an imidazole gradient (40 to 175 mM in 150 mL, 5 mL/min) in
phosphate buffer. Fractions (5 mL) were collected and those with \( \text{Abs}_{411}/\text{Abs}_{280} > 1.5 \) were pooled, concentrated and exchanged into Tris-HCl buffer (50 mM, pH 8.0) containing EDTA (0.5 mM) and NaCl (100 mM) by repeated centrifugal ultrafiltration (Centricon, 30,000 molecular weight cut-off, Millipore).

DTT was added to the resulting IDO1 solution to a final concentration of 1 mM followed by the addition of His-Tag TEV protease (~1 mg total). The reaction mixture was incubated overnight (4 °C) and then exchanged into imidazole-free phosphate buffer prior to elution over the Hi-Trap Chelating HP column to remove His-Tag TEV protease and any cleaved poly-His tag. This column was developed with an imidazole gradient (0 to 175 mM in 150 mL, 5 mL/minute). Fractions (5 mL) with \( \text{Abs}_{411}/\text{Abs}_{280} > 1.5 \) were pooled, concentrated and exchanged into Tris-HCl buffer (20 mM, pH 7.5) by centrifugal ultrafiltration. The resulting enzyme was purified further by passage over a column of Cibacron 3GA agarose 3000-CL resin (5 mL) equilibrated in the same Tris-HCl buffer. The enzyme was eluted with a sodium chloride gradient (0 to 500 mM in 50 mL), and fractions with \( \text{Abs}_{404}/\text{Abs}_{280} \geq 2 \) were pooled, concentrated and exchanged into Tris-HCl (20 mM, pH 8.0) containing EDTA (5 mM), NaCl (100 mM), and glycerol (20%) prior to freezing with liquid nitrogen for storage at −86 °C.

IDO1 purified by this procedure typically exhibited an \( \text{Abs}_{404}/\text{Abs}_{280} \) ratio of 2.05 in potassium phosphate buffer (100 mM, pH 7.5). Typical yield was approximately 15 mg of purified protein per litre of culture. In all cases, IDO1 concentrations were determined from molar absorptivity of the ferric form \( \varepsilon_{404nm} = 172,000 \text{ M}^{-1}\text{cm}^{-1} \) (Papadopoulou et al. 2005).

### 2.2.3 Recombinant human IDO2 and Δ27IDO2

Expression of IDO2 and Δ27IDO2 was carried out essentially as described for IDO1 with some modifications. Cultures of *E. coli* Rosetta(DE3)pLysS carrying plasmids His-TEV-IDO2 or His-TEV-Δ27IDO2 were grown (37 °C) in LB broth (1 L in 2 L Erlenmeyer flasks) containing
kanamycin (50 mg/L) and shaken (250 rpm) until the culture reached an OD$_{600}$ of 0.5 to 0.6, at which point the temperature and shaking speed were reduced to 20 °C and 150 rpm, respectively. After one hour, filter-sterilized IPTG was added to a concentration of 0.1 mM to induce protein expression. Cell harvest, lysis, and the initial elution over the Ni$^{2+}$-loaded HiTrap-Chelating HP column were carried out as described for IDO1 but with some minor changes. First, all buffers included glycerol (5 %) but were otherwise the same as those used in the purification of IDO1. Second, cell lysis and purification were performed at ~4 °C. Cooling of the Emulsi-Flex C5 cell disruptor was achieved by circulating ice-cold H$_2$O (5 min). Both IDO2 and Δ27IDO2 eluted as a single peak comprised predominantly of apo-protein (IDO2 devoid of porphyrin, Abs$_{411}$/Abs$_{280}$ < 0.3). All fractions containing the apo-protein were pooled, concentrated, and exchanged into imidazole-free phosphate buffer. The concentration of apo-protein was estimated from Abs$_{280}$ (1 mg/mL ≈ 1 absorbance unit) and adjusted to a concentration of ~50 µM.

Hemin solution was prepared immediately before use by dissolving hemin chloride (1 mg; Frontier Scientific, Logan, UT) in NaOH solution (100 µL, 0.1 M) and diluting to a concentration of 1 mM with phosphate buffer. This hemin solution was added to the apo-protein in a dropwise fashion to a final molar excess of 2:1. Following overnight incubation at 4 °C, imidazole was added (final concentration 20 mM) and excess hemin was removed by re-elution of the reaction mixture over the HiTrap-Chelating HP using the same conditions as before. Fractions with $A_{411}/A_{280} > 1.5$ were collected and exchanged into the appropriate buffer for reaction with TEV protease as described for IDO1. Subsequent steps (i.e., removal of TEV protease and chromatography over Cibacron 3GA resin) were conducted as for the purification of IDO1 except that the storage buffer was Tris-HCl (20 mM, pH 7.5) containing NaCl (500 mM) and glycerol (20%).
IDO2 and Δ27IDO2 prepared in this manner typically exhibited \( \text{Abs}_{404}/\text{Abs}_{280} \) values \( \geq 2.1 \) in potassium phosphate buffer (100 mM, pH 7.5). Typical yield of Δ27IDO2 was approximately 4 mg per litre of culture, and very limited quantity of IDO2 (< 1 mg) was recovered in soluble form; the vast majority of expressed protein remained insoluble following cell lysis. The molar absorptivity of the Δ27IDO2 variant was determined by the pyridine hemochromagen method (De Duve 1948) to be 178,500 M\(^{-1}\) cm\(^{-1}\) at 404 nm (pH 7.5), and this value was used for the determination of enzyme concentrations.

### 2.3 Spectroscopy

Electronic absorption spectra and kinetic traces were recorded with Cary 3E, 4000, or 6000i spectrophotometers with quartz cuvettes (1 cm pathlength; Hellma). Fluorescence emission spectra and kinetic traces were recorded with a Cary Eclipse spectrofluorimeter with in sub-micro fluorescence dual-paths quartz cuvettes (1×1 cm or 0.3×0.3 cm as indicated; Hellma). Rapid-scanning absorption spectra and kinetics were recorded with an Olis Model RSM-1000 stopped flow spectrophotometer fitted with a 2 cm pathlength flowcell or with a Biologic SFM-400 fitted with a 0.2 cm pathlength flowcell and TIDAS diode array (MMS-UV/500-1). Temperature control for the Cary 3E, Olis Model RSM-1000, and Biologic SFM-400 instruments was achieved with a circulating water bath while the Cary 4000, 6000, and Eclipse were equipped with Peltier devices.

### 2.4 Numerical analysis of data

Quantitative experimental results were generally analyzed by non-linear regression fitting of the data to the appropriate models with Origin 8.0 (OriginLab, Northampton, MA). This program uses an iterative procedure based on Levenberg-Marquardt algorithm to minimize the reduced chi-square values. The coefficients of determination from individual fitting analyses were > 0.95. The fitted parameters and corresponding standard errors are reported.
2.5 Characterization of the $\beta$-NADH-peroxidase-oxidase activity of IDO1

2.5.1 Reagents

Bovine superoxide dismutase (SOD), bovine liver catalase, $\beta$-NADH, $\beta$-NAD$^+$, 30% hydrogen peroxide solution, L- and D-tryptophan, and phenazine methosulfate were obtained from Sigma-Aldrich. High purity nitrogen (Praxair) was purified further by passage through a column of BASF R3-11 copper oxide catalyst that had been reduced by heating in the presence of a 92:8 mixture of N$_2$ and H$_2$ gases. Unless otherwise indicated, stock solutions of $\beta$-NADH were prepared the day of use, protected from light, and stored on ice. Stock solutions of catalase were prepared by washing the crystals repeatedly with de-ionized water to remove thymol preservative before dissolving to a final concentration of 2 mg/mL in potassium phosphate buffer (100 mM, pH 7.5). Where possible, reagent concentrations were determined spectrophotometrically from the following molar absorptivities: $\beta$-NADH and nicotinamide mononucleotide hydride (NMNH), $\varepsilon_{340\text{nm}} = 6,300$ M$^{-1}$ cm$^{-1}$ (McComb et al. 1976, Berger et al. 2005); catalase, $\varepsilon_{405\text{nm}} = 120,000$ M$^{-1}$ cm$^{-1}$ per monomer (Vlasits et al. 2007); L-/D-tryptophan, $\varepsilon_{280\text{nm}} = 5,470$ M$^{-1}$ cm$^{-1}$ (Marmorstein et al. 1987); H$_2$O$_2$, $\varepsilon_{240\text{nm}} = 43.6$ M$^{-1}$ cm$^{-1}$ (Noble & Gibson 1970); N-formylkynurenine, $\varepsilon_{321\text{nm}} = 3,750$ M$^{-1}$ cm$^{-1}$ (Shimizu et al. 1978). SOD was quantified on the basis of the activity stated by the manufacturer for the specific lot number.

2.5.2 IDO1-catalyzed $\beta$-NADH oxidation assays

The reduction and oxygenation of IDO1Fe$^{3+}$ upon addition of $\beta$-NADH and/or other reagents was monitored spectrophotometrically at 25 °C. Typically, reactions were initiated by addition of each reagent stock solution (10–20 µL) to solutions of 1–5 µM IDO1Fe$^{3+}$ (700 µL) in the desired buffers, in masked 1 mL quartz cuvettes. The solutions were mixed manually before acquiring spectra at regular time intervals or monitoring absorbance as a function of time at selected wavelengths. To monitor reactions under anaerobic conditions, dissolved O$_2$ was
removed from stock reagent and protein solutions either by sparging or flowing humidified N\textsubscript{2} over the surface of the solutions (≥ 30 min) before transferring the solutions into a N\textsubscript{2}-atmosphere in a glove box (Vacuum Atmospheres; O\textsubscript{2} content <1 ppm). The solutions to be mixed were placed in separate chambers of Thunberg cuvettes and sealed inside the glove box to permit mixing immediately prior to initiation of data acquisition with a spectrometer outside of the glove box.

### 2.5.3 IDO1-catalyzed L-Trp oxidation assays as supported by β-NADH

Solutions containing IDO1Fe\textsuperscript{3+} (1 µM) and β-NADH (200 µM) prepared in MOPS buffer (20 mM, pH 7.0) were incubated (20 °C) until the IDO1Fe\textsuperscript{3+} was fully converted to IDO1Fe\textsuperscript{3+−O\textsubscript{2}•−} before rapid mixing with an equal volume of L-Trp solution (15-2000 µM in the same buffer) with an Olis Model RSM-1000 stopped-flow spectrophotometer. Absorption spectra were collected at 1 kHz, and kinetic traces at selected wavelengths were extracted for analysis. Formation of N-FK was monitored continuously at 321 nm (Ishimura et al. 1970), and the rates of product formation were corrected for absorbance changes resulting from β-NADH oxidation and/or dilution as appropriate. In some cases, the absorbance changes were monitored at 503 nm to observe the oxidation of the protein following the addition of L-Trp.

### 2.5.4 Simulated docking of β-NADH to IDO1

To evaluate the manner in which β-NADH might bind to IDO1, a computational strategy was developed to simulate this interaction. The coordinates of the structure of PIM-bound IDO1 reported by Sugimoto (PDB accession 2D0T, (Sugimoto et al. 2006)) was used as the starting point. PIM and solvent molecules were deleted from the coordinate file, and missing polypeptide (residues 361-379 were not observed in the experimentally determined structure) was reconstructed with the program Modeller 9v8 (Sali & Blundell 1993, Fiser et al. 2000, Marti-Renom et al. 2000, Eswar et al. 2006). The resulting structure was optimized by molecular
mechanics using the Gromos96 ffG43a2 force-field (GROMACS 4.0.5, (Berendsen et al. 1995, Lindahl et al. 2001, Hess et al. 2008)). Briefly, the IDO1 structure was re-solvated in a periodic cubic box, and neutralized with Na\(^{+}\) counter-ions. After energy minimization by the steepest-decent method, the system was subjected to 50 picoseconds of isothermal (300 K) equilibration using the v-rescale thermostat and the particle mesh Ewald method (Essmann et al. 1995) for electrostatic calculations. All residue positions were restrained except for those that were missing from the original coordinates. Cut-off distances of electrostatic and van der Waals interactions were set to 0.9 and 1.4 nm, respectively. The energy-minimized structure was subsequently used in docking simulations with AutoDock 4.2 (Morris et al. 2009). \(\beta\)-NADH and Cibacron blue coordinates were obtained from the Hetero-compound Information Centre-Uppsala (Kleywegt & Jones 1998), and NMNH coordinates were generated using the structure of \(\beta\)-NADH as a template. All torsions were enabled in \(\beta\)-NADH, NMNH, and Cibacron blue, but IDO1 was treated as a rigid-body. Initially a docking space encompassing the entire protein was implemented to simulate blind docking. The goal of this approach was to identify regions of the IDO1 structure that favour interaction with \(\beta\)-NADH rather than determination of a single lowest-energy solution. Subsequently, these favourable regions were used to identify potential docking conformations solved for smaller docking volumes of \(\sim 60 \text{ nm}^3\) and more exhaustive search parameters. Lamarckian genetic searches (100) were performed for each site with the minimum number of energy evaluations set to 25,000,000. Selected conformations of IDO1 \(\beta\)-NADH-complexes associated with high-scores (\textit{i.e.}, greater likelihood of occurrence) as evaluated by AutoDock 4.2 were refined further by the energy-minimization procedure described above.
2.6 Characterization of the indole peroxygenase activity of IDO1

2.6.1 Reagents

Porcine liver esterase, glucose oxidase (*Aspergillus niger*), indole, 3-methylindole, 2-methylindole, 2,3-dimethyl-indole, 2-oxoindole, 3-methyl-2-oxoindole, 3-acetoxy-indole, indole-2,3-dione (isatin), D-glucose, lidocaine, D-mannitol, *meta*-chloroperoxybenzoic acid (*m*-CPBA; 77% w/w), peracetic acid (32% w/v), and cumene hydroperoxide (80% w/v) were from Sigma-Aldrich. Where possible, reagent concentrations were determined spectrophotometrically with the following molar absorptivities: indole, $\varepsilon_{276} = 5670 \text{ M}^{-1} \text{ cm}^{-1}$ (Ramachandran & Witkop 1964); 3-methyl-indole, $\varepsilon_{280} = 5160 \text{ M}^{-1} \text{ cm}^{-1}$ (*ibid*); 2-methyl-indole, $\varepsilon_{270} = 6500 \text{ M}^{-1} \text{ cm}^{-1}$ (*ibid*); 2,3-dimethyl-indole, $\varepsilon_{280} = 6360 \text{ M}^{-1} \text{ cm}^{-1}$ (*ibid*); indoxyl-3-acetate, $\varepsilon_{278} = 5500 \text{ M}^{-1} \text{ cm}^{-1}$ (this work). Porcine liver esterase and glucose oxidase were quantified on the basis of activities reported by the manufacturer for the specific lots of enzyme, and both were used without further purification.

2.6.2 IDO1-catalyzed indole oxidation assays

Generally, small reaction volumes (30 µL) and high reagent concentrations were used to prepare samples intended for immediate HPLC analysis while larger reaction volumes (0.5–1 mL) using appropriately diluted reagents were used to prepare reaction mixtures to be monitored spectrophotometrically. All concentrations reported are final unless otherwise indicated. Indole oxidation reactions (30 µL) were initiated by addition of H$_2$O$_2$ (0.5–6 mM) to a solution containing IDO1 (200 µM) and indole (2 mM) in Tris-HCl (20 mM, pH 7.5, 20 °C) and were stopped after 60 s by addition of ice cold methanol (50 µL) to a final concentration of 62% (v/v). The high enzyme concentration was selected to observe ~100 turnovers before protein inactivation and to ensure that the reaction was complete within 60s. The precipitated protein was removed by centrifugation at 3,000 g for 5 minutes at 4 °C. The clarified sample (20 µL)
was filtered (0.2 µm filter) and analyzed immediately by HPLC. For the oxidation of methylsubstituted indoles, the limited aqueous solubilities of these substrates required that they be dissolved initially in methanol and subsequently diluted into Tris-HCl buffer. Therefore, these reactions typically included ~1 to 2 % (v/v) methanol, and the concentrations of the substituted indole substrates 3-methylindole (1 mM), 2-methylindole (1 mM), 2,3-dimethylindole (0.2 mM) that could be assayed were limited to the values indicated. Correspondingly, [IDO1] was reduced to 50 µM in these reactions.

Other indole substrates assayed in this work included 2-oxoindole and 3-oxoindole. Because 3-oxoindole reacts spontaneously with dissolved O₂, solutions of this substrate were prepared anaerobically from 3-acetoxy-indole by de-esterification with porcine liver esterase (2 units/mL) immediately before use under anaerobic conditions (Barnett & Seligman 1951). Anaerobicity of the reagents and proteins was established essentially as described in section 2.5.2 with the exception of H₂O₂. Dissolved O₂ was removed from H₂O₂ solutions by repeated freeze-pump-thaw cycles on a Schlenk line. D-Glucose (10-100 mM) and glucose oxidase (24–47 units/mL) were included in some cases to scavenge traces of O₂ (Benesch & Benesch 1953). As before, reaction components were transferred into Thunberg cuvettes in the glove box for those reactions that were monitored spectrophotometrically. For anaerobic samples intended for HPLC analysis, the reaction products were collected in the glove box after precipitating the protein with N₂ purged methanol, but sample clarification and HPLC analysis were performed outside the glove box.

### 2.6.3 HPLC analysis of reaction products

Products of indole oxidation catalyzed by IDO1 were analyzed by reverse-phase HPLC (Beckman System Gold) fitted with an Alltech Prosphere HP C₁₈ 300 Å column (5 µm, 250 mm × 4.6 mm) and a diode array detector (1 Hz acquisition, 200–600 nm spectral window)). The
column was developed at 0.5 mL/min with gradient I (20 to 50% methanol in 30 min, 50 to 100% methanol from 30 to 35 min, and a final 10 min hold at 100% methanol). Products were identified by comparison of HPLC retention time, UV spectrum, and molecular mass with the corresponding values obtained for commercial compounds or from the literature. Product quantification was afforded by integration of HPLC peak areas with the Beckman GOLD software, standard solutions of known concentration, and molar absorptivities (indole, $\varepsilon_{278} = 6000 \text{ M}^{-1} \text{ cm}^{-1}$ (50% MeOH); 2-oxoindole, $\varepsilon_{248} = 7680 \text{ M}^{-1} \text{ cm}^{-1}$ (Cornforth et al. 1951); indole-2,3-dione, $\varepsilon_{242} = 22,500 \text{ M}^{-1} \text{ cm}^{-1}$; 3-oxoindole, $\varepsilon_{227} = 22,300 \text{ M}^{-1} \text{ cm}^{-1}$ (estimated following quantitative conversion from its precursor). The corresponding values used for o-formylamino-benzaldehyde and 3-hydroxy-2-oxoindole were based on values for N-FK ($\varepsilon_{321} = 3750 \text{ M}^{-1} \text{ cm}^{-1}$ (Shimizu et al. 1978)) and 2-oxoindole, respectively. All of these reagents were fully soluble at least up to 2 mM in methanol/Tris-HCl buffer (50% (v/v)). For fractionating the oxidation products of 3-methylindole, 2-methylindole, and 2,3-dimethylindole, gradient II was used (20 to 100% methanol in 24 min and a final 10 min hold at 100% methanol). Concentrations of the products formed in these latter reactions were not determined.

Analysis of the methanol-insoluble product derived from indole oxidation (indigo blue) was performed by TLC (EMD; silica gel 60 F254) in chloroform after quenching and and removal of the soluble reaction components and solubilization of the insoluble materials by ethyl acetate (50 μL to a 30 μL reaction).

### 2.6.4 LC-MS analysis of $^{18}$O incorporation

IDO-catalyzed oxidation of indole by hydrogen peroxide was conducted in air-saturated buffer as described above and initiated with either natural abundance H$_2$O$_2$ or ~90% $^{18}$O enriched H$_2^{18}$O$_2$ in H$_2^{16}$O (ICON Isotopes, Summit, NJ). ESI-MS analyses of $^{18}$O incorporation were carried out with an Agilent Model 6210 LCMS equipped with an electrospray ion source.
(capillary voltage 3 kV, fragmentor voltage 70 eV) and a quadrupole detector operating in the positive scan mode \((m/z\ 100\ or\ 130–300)\). Samples were introduced into the mass spectrometer by in-line HPLC (Agilent 1260 Infinity) fitted with the same column mentioned in section 2.6.3, and developed with the same \(\text{H}_2\text{O}/\text{MeOH}\) gradients compensated for an increased flow rate (0.8 mL/min). The time required from the initiation of reaction to injection was \(~8\) min. Stability of the \(^{18}\text{O}\) label incorporation was assessed by repeated analyses of the same samples (now in \(~60\%\ \text{MeOH}\) incubated at room temperature (3 h). Calculation of percent \(^{18}\text{O}\) incorporation was corrected for natural isotope abundance contributions based on a previously reported method (Mirgorodskaya et al. 2000).

## 2.6.5 Kinetics of indole oxidation

Solutions of IDO1 and indole (1 \(\mu\text{M}\) and 13–400 \(\mu\text{M}\), respectively, in 20 mM Tris-HCl buffer, pH 7.5) were mixed manually with \(\text{H}_2\text{O}_2\) to a final concentration of 25 \(\mu\text{M}–4\) mM in a dual path, masked cuvette (1 cm \(\times\) 1 cm), and indole consumption was monitored (in triplicate or greater) by fluorescence emission at 345 nm (excitation 280 nm) at 20 \(^\circ\text{C}\). The contribution of the products 2-oxoindole and 3-oxoindole to fluorescence emission under these conditions was negligible. Similarly, no fluorescence quenching was apparent with [2-oxoindole] \(\leq 200\ \mu\text{M}\). Non-linearity of the fluorescence response was corrected with a standard curve (\(i.e.,\) emission at 345 nm as a function of [indole]). Initial rates were determined with a logarithmic approximation method (Lu & Fei 2003), and kinetic parameters were obtained by non-linear regression fitting of the data.

## 2.6.6 Indole oxidation by organic peroxides

Commercial preparations of the \(m\)-CPBA (77% w/w) contain its corresponding free acid \((m\)-chlorobenzoic acid). The free acid was extracted from a solution of \(m\)-CPBA dissolved in ethyl ether (10 g in 100 mL) with five volumes of potassium phosphate buffer (0.2 M, pH 7.5)
(Aggarwal et al. 1998). The \( m \)-CPBA ether layer was then evaporated to dryness. Stock solutions of \( m \)-CPBA purified in this manner were prepared in acetonitrile and diluted in potassium phosphate buffer (200 mM, pH 7.5) to desired concentrations (~1% v/v acetonitrile) while peracetic acid was prepared directly in buffer. Reactions (30 \( \mu \)L) were initiated by addition of peracids to mixtures of IDO1 and indole (100 \( \mu \)M and 2 mM, respectively, in 200 mM potassium phosphate buffer, pH 7.5) to a final concentration of 4 mM, terminated after 30 s with ice-cold methanol (50 \( \mu \)L), and finally analyzed by HPLC as described in section 2.6.3. Changes in the spectrum of IDO1 during reaction with \( m \)-CPBA were recorded with the Biologic RSM-400 stopped-flow spectrophotometer after rapid mixing of the protein solution (20 \( \mu \)M) with an equal volume of the peracid (50 \( \mu \)M) in potassium phosphate buffer (200 mM, pH 7.5) at 10°C.

Solutions of IDO1\( \text{Fe}^{3+} \) or (sperm whale) metmyoglobin (500 \( \mu \)M protein in 200 mM potassium phosphate buffer, pH 7.5) were incubated with cumene hydroperoxide (5 mM) for 15 min and prepared for HPLC analysis as described above. Elution was carried out isocratically in 50% MeOH/H\( _2 \)O (0.5 mL/min). Concentrations of cumene alcohol were determined from the peak area using cumene hydroperoxide standards with the assumption that the two compounds exhibit comparable molar absorptivities (\( \varepsilon_{257\text{nm}} = 280 \text{ M}^{-1} \text{ cm}^{-1} \) in 50% MeOH/buffer). The concentrations of acetophenone, on the other hand, were based on a \( \varepsilon_{240\text{nm}} \) of 12,500 \( \text{ M}^{-1} \text{ cm}^{-1} \) (Forbes & Mueller 1955). The sum of the concentrations of the products (cumene alcohol and acetophenone) determined in this manner was approximately 96% of the starting substrate, which validated the assumption that molar absorptivity of the alcohol is approximately equivalent to that of the the hydroperoxide.
2.7 Characterization of the biochemical properties of IDO2

2.7.1 Reagents

For brevity, only reagents not already discussed in sections 2.5.1 and 2.6.1 are listed here. 2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), methylene blue, sodium ascorbate, horseradish peroxidase, glycerol (> 99.5% spectrophotometric grade) and riboflavin were from Sigma-Aldrich and were used as received. Stock solutions of ABTS and sodium ascorbate were prepared immediately before use and were stored in the dark and on ice. Where possible, reagent concentrations were determined spectrophotometrically: methylene blue, $\varepsilon_{664\text{nm}} = 95,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Bergmann & O'Konski 1963); riboflavin, $\varepsilon_{373\text{nm}} = 10,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Zondag et al. 1960).

2.7.2 Homology modeling of IDO2

The sequences of IDO1 and IDO2 were aligned with CLC Sequence Viewer 6.6.2 (CLC Bio A/S, Cambridge, MA). Automated modeling of the IDO2 structure was carried out with SwissPDB Viewer 4.0 (Arnold et al. 2006) at the ExPASy server based on this alignment (see Appendix B) and starting with chain A of PDB file 2D0T (Sugimoto et al. 2006).

2.7.3 Δ27IDO2-catalyzed L-Trp oxidation assays

The L-Trp dioxygenase activity of Δ27IDO2 was investigated using both chemical and enzymatic reduction methods. The chemical reducing system, as modified from the works of Yamamoto and Hayaishi (Yamamoto & Hayaishi 1967), consisted of ascorbic acid (20 mM), methylene blue (20 µM), and bovine liver catalase (50 µg/mL) in potassium phosphate buffer (100 mM, pH 7.5). The enzymatic reducing system consisted of tryptic cytochrome $b_5$ (10 µM), cytochrome $b_5$ reductase (150 nM), and β-NADH (150 µM) in Tris-HCl buffer (20 mM, pH 7.5). All reactions were performed at 20 °C. Solutions of Δ27IDO2 (400 µL, 0.5–1 µM) were incubated with the respective reducing system at 20 °C for 5 min prior starting the reaction by
addition of L-Trp solution (100 μL, 0.05–15 mM final concentrations). The progress of Ν-FK production under steady-state conditions was monitored at 321 nm, and the relevant Michaelis-Menten parameters were obtained by non-linear regression analysis of the data.

2.7.4 Photochemical reduction

The midpoint reduction potential \( (E_m) \) of the \( \Delta 27{\text{IDO2}} \, \text{Fe}^{3+}/\text{Fe}^{2+} \) couple was determined with a modified photochemical reduction technique first described by Massey and Palmer (Massey & Palmer 1966). Dissolved oxygen in all reagent and buffer solutions was purged with \( \text{N}_2 \) as described in section 2.5.2. Anaerobic solutions of methylene blue (10 μM) in potassium phosphate buffer \( (100 \, \text{mM, pH 7–8}) \) containing EDTA \( (10 \, \text{mM}) \) were transferred by cannula from sealed reaction vials to a sealed cuvette containing a concentrated solution of \( \Delta 27{\text{IDO2}} \) \( (10 \, \mu\text{L}) \). The transfer was performed under constant positive \( \text{N}_2 \) pressure to ensure anaerobicity and under subdued lighting conditions to avoid premature photochemical reduction of the protein prior to spectrum acquisition. Reduction of the protein was achieved by illuminating the sample with a 12 V halogen lamp, for short periods initially (~1–2 sec), and then for longer periods as required to achieve complete reduction of the heme iron. A water bath was used to suppress heating of the sample during photoirradiation. Spectra were recorded between exposures to monitor the progress of the reduction of heme at 404 nm and the reduction of methylene blue, which served as both a reporter and mediator, at 667 nm. Absorbance overlap between the protein and the dye at these wavelengths was negligible. This data was used to derive the midpoint potential \( (E_m) \) of the \( \Delta 27{\text{IDO2}} \, \text{Fe}^{3+}/\text{Fe}^{2+} \) couple by analysis using the Nernst equation.

\[
E = E_m - \frac{RT}{nF} \ln Q
\]  

(2.1)

Here \( RT/F \) (constant) is 58.2 mV at 20°C; \( E \) is the potential of the half-cell reaction corresponding to reduction of the ferric heme or methylene blue, as determined from the ratio \( Q \)
of the reduced to oxidized species (i.e., [IDO2Fe$^{3+}$/IDO2Fe$^{2+}$] or [oxidized methylene blue]/[reduced methylene blue]); $n$ is the number of electrons involved for the half-cell reactions. Values of Q were obtained on the basis of the absorbance changes of the spectra of Δ27IDO2 (404 nm) and methylene blue (665 nm) at various irradiation. The $E_m$ of the methylene-blue/leuco-methylene blue-couple was based on previously reported values of +11 mV at pH 7.0 versus standard hydrogen electrode (Merbitz-Zahradnik et al. 2003) and −250 mV versus saturated calomel electrode at pH 8.0 (Pessoa et al. 1997). Because there is only one potential for a given solution under equilibrium conditions, and $E_m$ of methylene blue (MB) is known, $E_m$ of Δ27IDO2 is thus determined from equation 2.2.

$$\frac{RT}{nF} \ln \left( \frac{[Fe^{3+}]}{[Fe^{2+}]} \right) = \frac{RT}{nF} \ln \left( \frac{[MB_{ox}]}{[MB_{red}]} \right) + 11 \text{ mV} - E_m(\text{IDO2}) \quad (2.2)$$

2.7.5 Auto-oxidation kinetics of the dioxygen-adduct of Δ27IDO2

The quantitative reduction of the ferric Δ27IDO2 was achieved by the photo-reduction method described in section 2.7.4 with some modifications. In these experiments, methylene blue was replaced by riboflavin (250 nM), and glycerol (5%) was included to stabilize the protein for extended periods of data acquisitions. Complete photoreduction of the heme iron was achieved by irradiating solutions of 5 μM protein (750 μL) in Bis-tris buffer (20 mM, pH 6.5–7.0) or Tris-HCl buffer (20 mM, pH 7.5–8.5) containing 5 mM EDTA with a 410 nm light-emitting diode (15–30 min). An equal volume (750 μL) of air-saturated buffer was added manually to the reduced protein to initiate acquisition of spectra at 1–3 min intervals, 20 °C. The air-saturated buffer included catalase (0.5 μg/mL) and SOD (1 unit/mL) to scavenge H$_2$O$_2$ and O$_2^-$ released during protein auto-oxidation. No apparent reduction of the protein (prior to mixing with buffer) was detected in the absence of ambient light or during repeated exposure to 700–450 nm light.
during spectrum acquisition. Absorbance at 577 nm was extracted from this family of spectra for kinetic analysis. Under these conditions, riboflavin contributed ~2% of the absorbance.

2.7.6 Δ27IDO2-catalyzed indole and ABTS oxidation assays

The kinetics of indole oxidation by Δ27IDO2 and H₂O₂ were determined essentially as described for IDO1 (section 2.6.5) with a few modifications. All reagents were prepared and diluted in Tris-HCl buffer (20 mM, pH 7.5) containing glycerol (5%). Reactions were initiated by the addition of H₂O₂ to mixtures of Δ27IDO2 and indole (800 nM and 4.5–630 μM, respectively) to final concentrations of 0.13–4 mM. Reaction mixtures (30 μL containing 50 μM protein, 500 μM indole and 1 mM H₂O₂) were prepared separately for product analysis by HPLC as described for IDO1 in section 2.6.3.

2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) is widely used as a substrate for the measurement of peroxidase activity because of its low midpoint potential relative to ferryl intermediates of peroxidase enzymes. Moreover, the intense absorption increase at 735 nm upon its oxidation to the cation radical (ABTS⁺) provides a sensitive means of detecting and monitoring peroxidase activity (Childs & Bardsley 1975). Reagents used in the oxidation of ABTS were prepared in the same buffer used in the oxidation of indole. Formation of ABTS⁺ was monitored by the absorbance changes at 735 nm following rapid mixing of Δ27IDO2 (300 nM), ABTS (0.1–7 mM), and H₂O₂ (1–40 mM), in that order, with the Biologic SFM-400 stopped-flow instrument fitted with a 4-syringe variable mixer and a 2 × 2 mm flow cell (FC–20). The concentrations of ABTS or H₂O₂ were varied by adjusting the volumes of reagents and buffer that were mixed. Dead time was estimated to be ~6 ms based on the flowrate. Initial rates of product formation were determined based on the change in absorbance at 735 nm by the ABTS cation radical (ε_{735nm} = 15 mM⁻¹ cm⁻¹, (Lu & Yeh 2011)).
Chapter 3: results

3.1 Properties of the aerobic reaction of β-NADH with IDO1

3.1.1 Aerobic conversion of IDO1Fe$^{3+}$ to IDO1Fe$^{3+}$–O$_2$•$^-$ in the presence of β-NADH

The aerobic addition of β-NADH to IDO1Fe$^{3+}$ in the absence of mediators resulted in increasing absorbance maxima at 577 and 542 nm (Figure 3.1 A) that are characteristic of the α- and β-transitions, respectively, in the spectrum of IDO1Fe$^{3+}$–O$_2$•$^-$ (Sono 1986). The observation of well-defined isosbestic points (410, 467, 527, and 595 nm) further indicated that other intermediates (i.e., IDO1Fe$^{2+}$) did not accumulate appreciably in this process. This reaction resulted in near quantitative conversion of IDO1Fe$^{3+}$ to IDO1Fe$^{3+}$–O$_2$•$^-$ as indicated by the virtual disappearance of the 633 nm charge transfer band of the high spin ferric heme and the greater intensity of the α-band ($\varepsilon_{577nm} = 12,970$ M$^{-1}$ cm$^{-1}$) relative to the β-band ($\varepsilon_{544} = 12,300$ M$^{-1}$ cm$^{-1}$). An alternative preparation of IDO1Fe$^{3+}$–O$_2$•$^-$ species was achieved by conventional anaerobic reduction of IDO1Fe$^{3+}$ by sodium dithionite, removal of the reducing agent, and finally, exposure of IDO1Fe$^{2+}$ to O$_2$. The spectrum of the protein obtained using this alternative approach was identical to that formed by aerobic reaction with β-NADH, and thus confirmed the identification of this enzyme derivative produced by aerobic exposure to β-NADH as IDO1Fe$^{3+}$–O$_2$•$^-$.  

In this reaction, the rate and extent of IDO1 oxygenation and the stability of the resulting IDO1Fe$^{3+}$–O$_2$•$^-$ product were dependent on the concentration and history (see section 3.1.5) of the β-NADH solution. Specifically, both the initial rates and the peak IDO1 oxygenation levels varied directly with β-NADH concentration (Figure 3.1 B). At lower [β-NADH], a relatively small fraction of the enzyme was maintained in the oxygenated state presumably reflecting a
Figure 3.1 Conversion of IDO1Fe$^{3+}$ to IDO1Fe$^{3+}$–O$_2$$^{•−}$ upon reaction with β-NADH under aerobic conditions. (A) Absorption spectra of IDO1Fe$^{3+}$ (1.5 μM protein in 20 mM MOPS, pH 7.0, 25 °C) before (initial bold curve) and every 2 min after addition of β-NADH (340 nm) to a final concentration of 250 μM. Arrows depict changes in absorbance spectrum of IDO1 as the reaction progresses. (B) IDO1Fe$^{3+}$–O$_2$$^{•−}$ formation as a function of β-NADH concentration monitored at 577 nm. The inset depicts the initial rates of IDO1Fe$^{3+}$–O$_2$$^{•−}$ formation.
steady-state equilibrium between protein auto-oxidation (i.e. conversion of $\text{IDO1Fe}^{3+} - \text{O}_2^{-}$ to the ferric state; $t_{1/2}$ values of $\text{IDO1Fe}^{3+} - \text{O}_2^{-}$ range from 36 s (Chauhan et al. 2008) to ~ 25 min (Hirata et al. 1977) for the human and rabbit isoform, respectively) and the continuing formation of $\text{IDO1Fe}^{3+-}\text{O}_2^{-}$. At $[\beta\text{-NADH}] > 750 \, \mu\text{M}$, near-quantitative oxygenation of IDO1 was achieved within minutes, but the apparent oxidation of $\text{IDO1Fe}^{3+} - \text{O}_2^{-}$ began sooner, and it proceeded at an accelerated rate in a pH-dependent manner (see section 3.1.4). In this case, the intensities of the $\alpha$- and $\beta$-bands decayed nearly linearly with time, but without restoration of the spectrum of $\text{IDO1Fe}^{3+}$. Instead, the spectrum exhibited increasing light scattering that is attributed to protein damage incurred through continued exposure of IDO1 to the reactive oxygen by-products or to side reactions involving the reactive oxygen species and $\beta$-NADH. Probable by-products of the oxidation of $\text{IDO1Fe}^{3+-}\text{O}_2^{-}$ include $\text{O}_2^{-}$ (Hirata et al. 1977) and, through dismutation of this reactive oxygen species, $\text{H}_2\text{O}_2$ (Sutton et al. 1976, Olek et al. 2002). The latter, when accumulated to sufficiently high concentrations, can have deleterious effects on IDO1 as previously reported (Hirata et al. 1977, Poljak et al. 2006).

### 3.1.2 Role of dioxygen

Anaerobic addition of $\beta$-NADH to $\text{IDO1Fe}^{3+}$ produced little or no spectroscopic change even after prolonged incubation although addition of catalytic amounts of phenazine methosulfate or methylene blue initiated rapid reduction of $\text{IDO1Fe}^{3+}$. In this case, the oxidation of $\beta$-NADH proceeded with the accumulation of $\text{IDO1Fe}^{2+}$ and the corresponding two-electron reduced form of the mediators as indicated by the disappearance of the absorbance bands of methylene blue (oxidized, $\lambda_{\text{max}} = 665 \, \text{nm}$) (Figure 3.2). Subsequent exposure of this reaction mixture to air led to rapid formation of $\text{IDO1Fe}^{3+} - \text{O}_2^{-}$, re-oxidation of methylene blue, accelerated consumption of $\beta$-NADH, and eventually, the auto-oxidation of the heme iron.
Figure 3.2 Role of O$_2$ in reaction of IDO1Fe$^{3+}$ with β-NADH. Absorption spectra of IDO1Fe$^{3+}$ observed under anaerobic conditions (6 μM in 20 mM Bis-tris, pH 7.0, 25 °C) upon addition of β-NADH (500 μM, initial bold), and 3 hr following addition of methylene blue (4 μM, final bold). The absorbance maximum at 665 nm corresponds to oxidized methylene blue when it is added initially. Arrows indicate the direction of absorbance changes as the reaction progresses. The inset depicts progress of IDO1Fe$^{3+}$ reduction as monitored by absorbance changes at 404 nm, following the addition of methylene blue at the indicated time point.

3.1.3 Effects of reactive oxygen species scavengers

Superoxide dismutase (SOD) inhibited the formation of IDO1Fe$^{3+}$–O$_2^{-\bullet}$ following exposure of the oxidized enzyme to β-NADH, and the extent of IDO1Fe$^{3+}$–O$_2^{-\bullet}$ formation varied inversely with SOD concentration. By comparison to the level of oxygenation achieved in the absence of SOD (Figure 3.3 A), only ~35 and 26 % oxygenation of IDO1 was achieved with 2.6 and 26 units SOD activity/mL of reaction ([SOD] ~50–500 nM), respectively (Figure 3.3 B). Interestingly, IDO1Fe$^{3+}$–O$_2^{-\bullet}$ formed in the presence of SOD reverted more rapidly to the ferric state (exponential absorbance decay at 577 nm compared to the nearly linear decay observed without SOD; Figure 3.3 B inset), and the auto-oxidation product formed under these conditions...
Figure 3.3 Role of superoxide anion radical ($O_2^-$) in aerobic reactions of IDO1Fe$^{3+}$ with β-NADH. Comparison of the aerobic reaction of 3.7 μM IDO1Fe$^{3+}$ with 500 μM β-NADH in the absence and presence of 2.6 units of SOD activity/ml of reaction (panels A and B, respectively). The insets illustrate the time-dependent changes in absorbance at 503 and 577 nm. The inset in (B) exhibits two 577 nm profiles that correspond to reactions carried out with 2.6 ($a$, ⋄) and 26 ($b$, ×) units of SOD activity/ml of reaction.
exhibited the spectrum of the native ferric state of the enzyme rather than the light scattering noted earlier. Presumably, dismutation of any \( \text{O}_2\cdot^- \) that was produced interfered with the reduction and oxygenation of IDO1Fe\(^{3+}\), but the total \( \text{H}_2\text{O}_2 \) produced in the process was not sufficient to damage the enzyme within the ~90 minute monitoring period.

Catalytic amounts of catalase also inhibited the rate and extent of IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) formation. When the two proteins were mixed together prior to addition of \( \beta\)-NADH, the efficiency of IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) formation decreased with increasing catalase concentration and, at sufficiently high [catalase], IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) formation is completely inhibited except for an initial and reversible reaction burst (Figure 3.4 A). Incubation of \( \beta\)-NADH with catalase before its addition to IDO1Fe\(^{3+}\) also resulted in diminished formation of IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\). The extent of catalase inhibition of this conversion under these conditions depends on the duration of the incubation period (Figure 3.4 B) and, as observed with SOD present in the solution, any IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) that accumulated quickly reverted to the ferric state. In fact, addition of catalase to IDO1 and \( \beta\)-NADH mixtures at any early stage of the reaction accelerated the oxidation of the protein to a state with the spectrum characteristic of the ferric protein. In contrast, addition of small amounts of \( \text{H}_2\text{O}_2 \) to IDOFe\(^{3+}\) solutions that had reached a low but stable level of oxygenation by exposure to a low concentration of \( \beta\)-NADH resulted in a prompt increase in the rate of IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) formation (Figure 3.4 C). This enhancement in IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) formation increased with [\( \text{H}_2\text{O}_2 \)], but the protein was also more susceptible to auto-oxidation and the protein damage that followed.

3.1.4 pH-Dependence

The pH-dependence of IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) formation in the presence of \( \beta\)-NADH exhibited features that were consistent with the involvement of O\(_2\)\(\cdot^-\) (Figure 3.5 A). Both the initial rate of oxygenation and the maximum amount of IDO1Fe\(^{3+}\)–O\(_2\)\(\cdot^-\) formed increased with pH to
Figure 3.4 Influence of H$_2$O$_2$ on the reactions observed upon aerobic addition of β-NADH to IDO1Fe$^{3+}$. IDO1Fe$^{3+}$ (4 μM in 20 mM Bis-tris, pH 7.0, 25°C) conversion to IDO1Fe$^{3+}$–O$_2$•− monitored at 577 nm by (A), freshly dissolved β-NADH (800 μM) in the presence of bovine liver catalase (0–230 nM as shown) or (B), 4 days old β-NADH (incubated at pH 7.0 in the dark and on ice) and treated with bovine liver catalase (500 pM) for the times indicated (1–96 minutes) prior to its addition to IDO1Fe$^{3+}$ (final [NADH] 205 μM). (C) Enhancement of IDO1Fe$^{3+}$–O$_2$•− formation by reaction with freshly dissolved β-NADH (25 μM) followed by addition of H$_2$O$_2$ (0–2 μM as indicated) at the timepoint indicated by the arrow.
Figure 3.5 Dependence of aerobic reaction of IDO1Fe³⁺ with β-NADH on pH. (A) Aerobic reaction of 2 µM IDO1Fe³⁺ with 250 µM β-NADH at the pH indicated (20 mM Bis-tris for pH 6.0–7.0; 20 mM Tris-HCl for pH 7.5–8.0; 20 mM TAPS for pH 8.5–9.0), and monitored by the change in absorbance at 577 nm. (B) Extent of IDO1Fe³⁺→O₂−•− formation normalized to the maximum ΔAbs 577 nm. Fitting of these data to a three-state, two-deprotonation model yielded pKₐ values of 6.5 ± 0.2 and 8.6 ± 0.5. (C) Rates of IDO1Fe³⁺→O₂−•− formation based on initial slopes of absorbance changes. (D) Rates of IDO1Fe³⁺→O₂−•− oxidation based the slopes of absorbance changes ~20 min after β-NADH addition. Fitting these data to a two-state single deprotonation model yielded a pKₐ of 6.8 ± 0.1. Error bars denote the standard deviation of measurements performed in triplicate.
maximum values at pH ~7.8 (Figure 3.5 B and C, respectively). At pH > 8.5, the extent of oxygenation was markedly reduced, but the resulting IDO1Fe\(^{3+}\)-O\(_2\)\(^{•-}\) was more stable. Fitting these data to a three state, two-deprotonation model yielded pK\(_a\) values of 6.5 ± 0.2, and 8.6 ± 0.5. The rate constants for the reaction of O\(_2\)\(^{•-}\) (generated *in situ* via pulse radiolysis) with IDO1Fe\(^{3+}\) reportedly increase monotonically with decreasing pH (Kobayashi *et al.* 1989). In contrast, the yield of IDO1Fe\(^{3+}\)-O\(_2\)\(^{•-}\) formed following exposure to β-NADH was lower at pH < 7.5, which may result from a reduced supply of O\(_2\)\(^{•-}\) that can be attributed to the more efficient dismutation of free O\(_2\)\(^•\) to H\(_2\)O\(_2\) at lower pH (Bielski & Allen 1977). Moreover, the rate of auto-oxidation of IDO1Fe\(^{3+}\)-O\(_2\)\(^{•-}\) likely increased at lower pH, as observed for myoglobin and hemoglobin (Brantley *et al.* 1993, Tsuruga *et al.* 1998). As a result, the onset of light-scattering in the spectrum of the protein occurred significantly earlier in reactions carried out at lower pH. The decay rates of the α-band (absorbance 577 nm) following peak oxygenation as a function of pH were fitted to a two-state, single-deprotonation model to yield a pK\(_a\) of 6.8 ± 1 (Figure 3.5 D)

### 3.1.5 Quality of β-NADH

The rate and extent of reaction following aerobic exposure of IDO1Fe\(^{3+}\) to β-NADH increased significantly with the age of the β-NADH solution. Incubation of a stock β-NADH solution (pH 7.0) in the dark at room temperature for two hours, for example, nearly doubled the yield of IDO1Fe\(^{3+}\)-O\(_2\)\(^{•-}\) observed following exposure of the enzyme to a freshly prepared solution of β-NADH, and this difference increased with time beyond 96 hours of incubation (Figure 3.6 A). Enhancements in the rate of IDO1Fe\(^{3+}\)-O\(_2\)\(^{•-}\) formation were also observed with β-NADH solutions that were incubated on ice or with freshly prepared solutions that had been heated for 5 minutes at 95 °C. Known aerobic decomposition products of β-NADH include β-NAD\(^+\), H\(_2\)O\(_2\), and/or a β-NADH-peroxide complex (Dolin 1962, Bernofsky 1987). Presumably,
[H₂O₂] correlated with the duration β-NADH was exposed to atmosphere, and as a result, the rates of the reaction were not always reproducible among various stocks of the reducing agent. This conclusion is consistent with the earlier observation that β-NADH solutions incubated with catalytic amount of catalase had significantly diminished reactivity with IDO1Fe³⁺.

3.1.6 IDO1Fe³⁺–O₂⁻ formation in the presence of reduced nicotinamide mononucleotide

Exposure of IDO1Fe³⁺ to aerobic solutions of α-NADH, and β-NADPH also resulted in formation of IDO1Fe³⁺–O₂⁻. Because of the variability resulting from the age and condition of these reducing agents mentioned above, the rates of IDO1Fe³⁺–O₂⁻ formation are not directly comparable for different stock solutions. For example, the ratio of the absorbance intensities at 260 nm (oxidized) to those at 340 or 347 nm (reduced) was 2.43 and 2.57 in freshly prepared solutions β-NADPH and α-NADH, respectively, indicating that the extent of oxidation and by extension, H₂O₂ accumulation, was not the same for the two solutions. As a result, to achieve similar levels of IDO1Fe³⁺–O₂⁻, the reactions required approximately two-fold greater concentrations of β-NADPH than α-NADH.

On the other hand, reactions carried out with nicotinamide mononucleotide (NMNH) that had been prepared by hydrolysis of β-NADH (same stock solution throughout) with phosphodiesterase I resulted in a significant increase in the rate and extent of IDO1Fe³⁺–O₂⁻ formation (Figure 3.6 B). The amount of IDO1Fe³⁺–O₂⁻ formed correlated initially with the duration of hydrolysis (i.e., [NMNH]). However, oxidation of the enzyme to the ferric state also occurred earlier and at a faster pace commensurate with the length of time that the β-NADH was subjected to phosphodiesterase cleavage. This observation indicated that IDO1Fe³⁺–O₂⁻ formation was initially greater due to higher H₂O₂ content, presumably resulting from the
relatively greater reactivity of NMNH with dioxygen, but excess H$_2$O$_2$ accumulation and depletion of the reducing agents eventually decreased reactivity.

Figure 3.6 Aerobic reactions of IDO1Fe$^{3+}$ with various preparations of β-NADH. IDO1Fe$^{3+}$ (5.6 μM in 20 mM Bis-tris, pH 7.0, 25°C) conversion to IDO1Fe$^{3+}$–O$_2$•$^-$ monitored at 577 nm by β-NADH (200 μM) that had been incubated in the dark for 0, 2, 5, 8, 24, 28, and 96 h (t0–t6, respectively) at 23 °C, in absence and presence of snake venom phosphodiesterase I, panels (A) and (B), respectively. A β-NADH stock (50 mM in 100 mM Tris-HCl buffer, pH 8.9, 14 mM MgCl$_2$, and 100 mM NaCl) was divided into two portions (0.5 ml) for addition of 0.5 units of phosphodiesterase concentrate (5 μl) to one stock. Samples from the two stocks were withdrawn at the times indicated and frozen immediately in liquid nitrogen until shortly before assaying the activity of each.
3.1.7 Effects of IDO1 ligands

Addition of 4-phenylimidazole (PIM) to IDO1Fe$^{3+}$--O$_2$•$^-$ formed in the presence of β-NADH increased the rate of protein auto-oxidation. The resulting enzyme product exhibited the same spectroscopic features ([Figure 3.7 A](#)) reported for PIM-bound IDO1Fe$^{3+}$ (Sono & Cady 1989), which are those of a 6-coordinate, low-spin, ferric heme protein. In this case, the decay of IDO1Fe$^{3+}$--O$_2$•$^-$ (as monitored at the α-band) followed nearly first-order kinetics, and the apparent rate constant ($k_{obs}$) varied in proportion with [PIM], reaching a maximum (2.7 × 10$^{-3}$ sec$^{-1}$) at ~500 μM. During this period of decay, very little β-NADH was consumed. The maximal rate of IDO1Fe$^{3+}$--O$_2$•$^-$ decay was similar to that observed in the absence of reducing agents (1.6 × 10$^{-3}$ sec$^{-1}$). On the other hand, [PIM] at half-maximal $k_{obs}$ was 13 ± 2 μM ([Figure 3.7 B](#)), which is in close agreement with the reported values for the dissociation constant for binding of PIM to IDOFe$^{3+}$ ($K_d$ 10 μM, (Sono & Cady 1989)). Evidently, phenylimidazole competed with reactive oxygen species (*i.e.*, O$_2$•$^-$ and H$_2$O$_2$) in the coordination to the heme iron thereby interfering with the auto-catalytic nature of IDO1Fe$^{3+}$--O$_2$•$^-$ formation in the presence of β-NADH. This observation is also supported by the inhibitory effect of PIM on the O$_2$•$^-$-dependent oxidation of L-Trp by IDO1Fe$^{3+}$ (Sono & Cady 1989).

Addition of indoleethanol to IDO1Fe$^{3+}$ and β-NADH also resulted in similar increases in the rate of IDO1Fe$^{3+}$--O$_2$•$^-$ oxidation ([Figure 3.7 C](#)). In this case, the maximal value of $k_{obs}$ for the conversion of IDO1Fe$^{3+}$--O$_2$•$^-$ to IDO1Fe$^{3+}$ (1.0×10$^{-2}$ sec$^{-1}$) was greater than that obtained in the presence of PIM, while [indoleethanol] required to reach half-maximal $k_{obs}$ was 680 ± 60 μM ([Figure 3.7 D](#)). This enhanced rate of protein auto-oxidation may be related to the proposed ability of indoleethanol to bind at an unknown allosteric site (Sono 1989). Specifically, indoleethanol binding to IDO1Fe$^{3+}$--O$_2$•$^-$ may promote heme oxidation directly, and/or prevent...
Figure 3.7 Effects of IDO1 ligands on IDO1Fe$^{3+}$–O$_2$$^-$ formed by aerobic reaction with β-NADH.
Figure 3.7 Effects of IDO1 ligands on IDO1Fe$^{3+}$–O$_2^{•−}$ formed by aerobic reaction β-NADH. Absorption spectra of IDO1Fe$^{3+}$–O$_2^{•−}$ formed by reaction with β-NADH (2 µM protein, 100 µM β-NADH in 20 mM MOPS, pH 7.0, 25°C) before (initial bold curve) and every 2 min after addition of (A) 4-phenylimidazole to a final concentration of 2 mM, or (B) indoleethanol to a final concentration of 1 mM. The arrows indicate the direction of the change in absorbance as the reaction progresses. The insets depict absorbance changes at 577 nm, which were fitted to a single exponential to obtain the decay rate constants of IDO1Fe$^{3+}$–O$_2^{•−}$ ($k_{obs}$); lower panels show residual plots from the fit. The dependence of $k_{obs}$ on the concentrations of 4-phenylimidazole and indole-ethanol are shown in panels (C) and (D), respectively.

Oxygenation of the protein. The latter proposal is consistent with the reported lower reactivity of O$_2^{•−}$ with IDO1Fe$^{3+}$ in the presence of indoleethanol (Kobayashi et al. 1989). It is known, however, that indoleethanol increases the rate of L-Trp oxidation by IDO1 (Sono 1989) in the standard steady-state activity assay that is supported by ascorbate and methylene blue. This enhanced enzyme activity by indoleethanol can be reconciled with its inhibitory effects on IDO1Fe$^{3+}$–O$_2^{•−}$ formation if indoleethanol binding also influences the conformation of the IDO1 ternary complex and/or favours activation of the enzyme through electron transfer in the presence of a mediator such as methylene blue.

3.1.8 Oxidation of L-Trp by IDO1 in the presence of β-NADH

Reaction of β-NADH-generated IDO1Fe$^{3+}$–O$_2^{•−}$ with L-Trp resulted in the production of N-FK based on increasing absorbance at 321 nm (Figure 3.8 A). The substrate turnover was initially ~3 s$^{-1}$, but this rate decreased monotonically to 0.02 s$^{-1}$ within 20 s. During the initial burst period (phase 1), only ~19 mole-equivalents of N-FK accumulated despite the availability of β-NADH and substrate in high excess. After this point, the oxidation reaction continued at a base level (phase 2, vide infra) until the L-Trp concentration became limiting. The properties of phase 1 paralleled those observed by Hayaishi and co-workers who observed a finite turnover of L-Trp oxidation reactions (~50 mole-equivalents of N-FK) in which the active enzyme was uncaged by flash photolysis of the IDO1Fe$^{2+}$–CO complex in oxygen-saturated buffer.
(Taniguchi et al. 1979). Similar results were also reported in analogous stopped-flow studies involving the ferrous enzyme (Lewis-Ballester et al. 2009).

In the studies above, N-FK production ceased following consumption of reducing agents that were used to maintain the enzyme in the active ferrous form so that IDO1Fe$^{3+}$ and/or IDO1Fe$^{3+}$–L-Trp accumulated. In the case of β-NADH-initiated reactions, the absorbance changes at 503 nm corresponding to the high-spin IDO1Fe$^{3+}$ indicated cycling between protein oxidation and re-oxygenation with each addition of 6 μM L-Trp (aliquoted additions at every ~20 s to minimize IDO1Fe$^{3+}$–Trp formation) (Figure 3.8 B). In the process, ~100 turnovers occurred before complete oxidation of the protein as indicated by the nearly complete recovery of the 503 nm absorbance band. At higher [L-Trp], the absorption changes of the protein in the visible region of the spectrum instead showed the formation of an IDO1Fe$^{3+}$–L-Trp-like species at the end of phase 1 (~20 s, 539 and 568 nm and a shoulder at ~593 nm, isosbestic points at 584 and 523 nm, Figure 3.8 D).

The rate of conversion of the ternary complex IDO1Fe$^{3+}$–O$_2$–L-Trp to IDO1Fe$^{3+}$ can be correlated with the rate of N-FK production observed during phase 1, and the product concentration [P] can then be expressed in terms of equation 3.1 (Taniguchi et al. 1979).

$$[P] = \frac{k_1[E]}{k_2} (1 - e^{-k_2t})$$

(3.1)

where $k_1$ and $k_2$ correspond to the apparent rate constants for product formation, and enzyme inactivation through oxidation to IDO1Fe$^{3+}$, respectively, while [E] corresponds to the initial concentration of the ternary complex. [E] used for this calculation was approximately equivalent to the concentration of IDO1Fe$^{3+}$–O$_2$– (1 μM) based on the rapid formation of the substrate bound complex, which was complete within 200 ms of mixing (isosbestic points at 572, 529, and 506 nm; Figure 3.8 C). The reaction was pseudo-first order with 10-fold excess L-Trp over
Figure 3.8 Phase 1 of IDO1-catalyzed oxidation of L-Trp as supported by β-NADH. (A) N-formylkynurenine formed (based on absorbance changes monitored at 321 nm with a stopped-flow spectrometer) by reaction of β-NADH-generated IDO1Fe$^{3+}$–O$_2^{-}$ (1 μM protein, 200 μM β-NADH in 20 mM MOPS buffer, pH 7.0, 20 °C) with L-Trp (a, 100 μM; b, 2 mM). (B) Conversion of IDO1Fe$^{3+}$ to IDO1Fe$^{3+}$–O$_2^{-}$ following the addition of β-NADH (2.5 μM protein, 1 mM β-NADH in the same buffer) and then back to IDO1Fe$^{3+}$ following addition of L-Trp (~6 μM per 20s, 250 μM total), as monitored at 503 nm. (C) and (D), time-dependent spectra of the visible region of β-NADH-generated IDO1Fe$^{3+}$–O$_2^{-}$ (15 μM) during the reaction with L-Trp (2 mM) in 0 to 200 ms, and 0.2 to 20 s. Arrows indicate the direction of the absorbance changes. Numbers above the arrows and numbers in parentheses denote positions of maxima and isosbestic points, respectively.

enzyme. N-FK production followed first order kinetics, and fitting the kinetics of N-FK formation to equation 3.1 (Taniguchi et al. 1979) yielded $k_1$ and $k_2$ values of 3.2 ± 0.3 and 0.17 ± 0.02 s$^{-1}$, respectively. The rate constant obtained for L-Trp turnover ($k_1$) was in reasonable agreement with the value reported by Taniguchi and co-workers (2.0 s$^{-1}$, (Taniguchi et al. 1979))
and with $k_{\text{cat}}$ values reported for this enzyme (3.1 ± 0.2 s$^{-1}$, (Lu et al. 2009)). The rate constant for the oxidation of the enzyme ($k_2$), on the other hand, was significantly greater than the corresponding value in the same report (0.028 s$^{-1}$, (Taniguchi et al. 1979)). This discrepancy may arise from the lower pH employed in the current work (7.0 versus 8.0), but reactive oxygen species involved in the reactions with $\beta$-NADH may also contribute significantly to the accelerated oxidation of the protein.

Nevertheless, the auto-oxidation of IDO1Fe$^{3+}$–O$_2$•$^-$ in the absence of substrate and reducing agents (i.e. no active turnover) was considerably slower (1.9 × 10$^{-2}$ to 16 × 10$^{-3}$ s$^{-1}$ estimated from $t_{1/2}$ values of 36 s$^{-1}$ (Chauhan et al. 2008) and ~7 min in this study, respectively), indicating that IDO1 is more susceptible to oxidation during turnover and/or while involved in the L-Trp complex. It is worth noting that similar rate constants were obtained at 100 μM and 2 mM L-Trp (Figure 3.8 A) and that substrate inhibition kinetics normally observed at steady-state were not detected during phase 1 for [L-Trp] ≤ 2 mM.

Any enzyme oxidation that resulted during L-Trp turnover was reversible. As L-Trp was consumed, the rate of $N$-FK production increased again in the late stages of phase 2, suggesting that oxidized enzyme (a mixture of IDO1Fe$^{3+}$ and IDO1Fe$^{3+}$–L-Trp) can be reactivated by the $\beta$-NADH remaining in the solution. Similarly, when L-Trp addition to solutions of IDO1Fe$^{3+}$ preceded enzyme reduction by addition of $\beta$-NADH, only phase 2 of the reaction was observed initially, but the rate of $N$-FK production increased nearly ten-fold as the [L-Trp] fell below ~30 μM (Figure 3.9 A). Because this threshold concentration was substantially below the inhibition constant cited by others for substrate inhibition of IDO ($k_{\text{si}}$ 170 μM, (Lu et al. 2009)), it would appear that if loss of enzyme activity at > 30 μM [L-Trp] was an issue in the substrate-triggered inactivation of the protein here, then its contribution was insignificant. Similarly, with a $K_d$ of
Figure 3.9 Phase 2 of IDO1-catalyzed oxidation of L-Trp as supported by β-NADH. (A) Typical reaction trace for phase 2 of L-Trp oxidation by IDO1 (1 μM protein in 20 mM MOPS buffer, pH 7.0, 20 °C) following depletion of the initial β-NADH-generated IDO1Fe$^{3+}$−O$_2$$. Initial rates (s$^{-1}$) of N-formylkynurenine formation (closed circles) and the fraction of reduced enzymes (open circles) as a function of the concentrations of L-Trp and D-Trp, panels (B) and (C) respectively. The fraction of reduced enzyme was estimated from the ratio of the observed initial rates to the rates of the fully reduced enzyme as defined by the Michaelis-Menten equation.

0.9 mM (Lu et al. 2010) for the binding of L-Trp by IDO1Fe$^{3+}$, only a small fraction of the ferric protein was actually present as the low-spin, IDO1Fe$^{3+}$−L-Trp complex. Therefore, L-Trp appears to exert its influence primarily through the promotion of the oxidation of the heme iron during turnover. The fraction of reduced enzyme as a function of L- and D-Trp concentration is shown in Figures 3.9 B and C, respectively. These results indicate that at low [L-Trp] (<< $K_m$), a
high fraction of enzyme remained in the oxygenated state as the L-Trp oxidation reaction proceeded, but increasing [L-Trp] resulted in an exponential decrease in the availability of IDO1Fe$^{3+}$–O$_2^•−$. While similar results were obtained with D-Trp, complete conversion to the ferric enzyme occurred at a much greater [D-Trp], which is consistent with the higher $K_m$ for this substrate.

3.1.5 Simulated docking of $\beta$-NADH to IDO1

Attempts to identify interaction site(s) on IDO1 for $\beta$-NADH, NMNH, and the dye Cibacron blue 3GA, which has structural similarity to the dinucleotides, were made with the program AutoDock 4.2 (Morris et al. 2009). These simulations yielded three potential interaction sites (Figure 3.10 A) all of which were electropositive in character suggesting that ionic interactions between basic residues (i.e. Lys116, Lys186 and Arg231 at the center of sites 1, 2 and 3, respectively), and the (pyro)phosphate or sulfonate groups of $\beta$-NADH/NMNH and Cibacron blue 3GA, respectively, may play important roles in stabilizing ligand binding to the enzyme. All three ligands clustered within two of the interaction sites (sites 1 and 2), but only a few complexes were found with Cibacron blue 3GA within the heme cavity, where $\beta$-NADH and the mononucleotide formed a third cluster (site 3).

In the complex with the highest docking value, $\beta$-NADH is modeled in a manner similar to the 4-phenylimidazole-IDO1 complex (Sugimoto et al. 2006). The nicotinamide ring is poised perpendicularly above the heme plane with ~4.7 Å separating the pyridyl C$_3$ carbon and the heme iron, where it appears to be held in position by cation-π interaction with Phe163. Hydrogen bonding interactions are predicted for the pyrophosphate group with Arg231 and Ser235, the nicotinamide amide group with Gly262, and the ribose group of adenine with Gly236 (Figure 3.10 B). A similar arrangement has been observed in the crystal structure of cytochrome P450 nitric oxide reductase (Oshima et al. 2004) with bound $\beta$-NADH analogue. In other
Figure 3.10 Potential β-NADH-IDO1 interaction sites predicted with AutoDock 4.2. (A) Sites of interaction are represented by the centers of mass of β-NADH (green), NMNH (magenta), and Cibacron blue 3GA (yellow) calculated from their respective predicted conformations and subsequently superimposed on an electrostatic potential surface map of IDO. Regions 1–3 represent areas of the IDO1 surface at which interaction with β-NADH, NMNH, or Cibacron blue is predicted to be most probable. (B) Structure of the highest-scoring docked complex of β-NADH and IDO1.
IDO1–β-NADH complex models, the adenine group appears at the distal side of the heme instead of the nicotinamide ring. Steric restrictions appear to prevent generation of models in which both adenine and the pyridine ring occupy the heme cavity simultaneously. Models with NMNH bound at the heme site exhibit significantly less variability because of the lack of steric restrictions associated with fitting the adenine moiety in the heme cavity, which suggests that the mononucleotide is better able to access the active site of the enzyme and thereby to promote reduction of the heme iron.

3.2 Properties of the reaction of indole, H$_2$O$_2$, and IDO1

3.2.1 IDO1Fe$^{3+}$-catalyzed indole(s) oxidation by H$_2$O$_2$

Addition of H$_2$O$_2$ to IDO1Fe$^{3+}$ resulted in the formation of a higher oxidation state of the protein, as indicated by a shift in the Soret maximum from 404 to 415 nm and the appearance of an absorbance band at 548 nm with shoulders at ~580 and 525 nm (Figure 3.11) as reported by Lu and co-workers who also identified this species as a ferryl intermediate (i.e. IDO1Fe$^{4+}$=O) based on a simultaneous resonance Raman analysis (Lu & Yeh 2011). This transition occurred with one set of isosbestic points (412, 462, 514 and 616 nm) in the first ~200 ms leading up to the presumed IDO1Fe$^{4+}$=O intermediate, suggesting that other intermediates did not accumulate appreciably under these conditions (5 or 30 µM protein, 2 mM H$_2$O$_2$). The pseudo-first order rate constant of this reaction was 17 s$^{-1}$, also in close agreement with that reported by Lu and co-workers. Following these initial absorbance changes, the Soret band decayed rapidly but without the recovery of the initial IDO1Fe$^{3+}$ spectrum.

The same reaction carried out in the presence of indole resulted in markedly diminished absorbance changes with the spectrum of the protein retaining predominantly ferric character (Figure 3.11). Nevertheless, the near isosbestic points and direction of absorbance changes under these conditions reflected partial accumulation of the ferryl heme and indicated reduction
Figure 3.11 Conversion of IDO1Fe$^{3+}$ to IDO1Fe$^{4+}$=O upon addition of H$_2$O$_2$. Absorption spectra of IDO1Fe$^{3+}$ (5–30 µM protein in 20 mM Tris-HCl, pH 7.5, 15°C) before and every 50 ms after addition of H$_2$O$_2$ to a final concentration of 2 mM, in the absence of indole (solid curve) and 200 ms after H$_2$O$_2$ addition in the presence of 2.5 mM indole (dotted curve). The arrows in indicate the direction of absorbance changes as the reaction progresses. The inset depicts changes in absorbance at 548 nm replotted against time (a, no indole; b, 2.5 mM indole).

of that species by indole. A commensurate decrease in the absorbance at 276 nm and a corresponding increase in the absorbance at ~233, 390, and 610 nm (Figure 3.12) were consistent with the oxidation of indole. This reaction proceeded with intense fluorescence emission (470 nm emission, 380 nm excitation). The appearance of the 610 nm band in particular was delayed relative to the other absorbance changes, and it continued to increase in intensity with time, eventually leading to the precipitation of blue-coloured substance(s). The lack of
isosbestic points in this family of spectra indicated that multiple products and/or intermediates were formed during the reaction. Accordingly, at least seven products were identified by HPLC analysis (Figure 3.13). Only the peaks with retention times corresponding to Tris-HCl buffer (7.5 min) and unreacted indole (35.5 min) were detected when either H₂O₂ or the enzyme were omitted in control reaction mixtures or when the enzyme was heat-denatured prior to reaction. Peak asymmetry (ratio of back peak width to front peak width at 10% peak height) was generally in the range of 0.8–0.85 with the exception of peaks IV and VI, which could not be resolved cleanly under the elution conditions employed.

![Figure 3.12](image)

**Figure 3.12** IDO1Fe³⁺-catalyzed oxidation of indole by H₂O₂. Absorption spectra of a solution mixture of indole and IDO1Fe³⁺ (248 μM indole, 700 nM protein in 20 mM Tris-HCl, pH 7.5, 20°C), following addition of H₂O₂ to a final concentration of 1 mM. Spectra in the UV and near-UV regions were recorded at intervals of 15 s (T₀, T₁, T₂, T₃ denote 0, 15, 30, 150 s, respectively). Spectra in the visible region were recorded at intervals of ~1.5 min (t₀, t₁, t₂, t₃ denote 0, 7, 10, 13 min, respectively). The 610 nm absorbance band corresponded to indigo blue, which continued to form even after the reaction terminated (see section 3.2.1). The arrows indicate the direction of absorbance changes overtime.
Figure 3.13 Products of IDO1Fe$^{3+}$-catalyzed oxidation of indole by H$_2$O$_2$. (A) HPLC analysis (gradient I; see section 2.6.3) of the products (I–VII) of the aerobic reaction of IDO1Fe$^{3+}$ with indole (200 μM protein and 2 mM indole in 20 mM Tris-HCl pH 7.5, 25°C) following addition of H$_2$O$_2$ to a final concentration of 4 mM. Numbers above the peaks denote retention times (0.5 mL/min flowrate). Protein/Tris buffer and unreacted indole elute at 7.5 and 35 min, respectively. Assignments of I–VII are listed in Table 3.1. (B) HPLC retention times (gradient I) of standards of (a) 3-oxoindole, (b) 3-hydroxy-2-oxoindole, (c) indole-2,3-dione, (d) 2-oxoindole, and (e) indole. Standards of 3-oxoindole and 3-hydroxy-2-oxoindole were prepared by anaerobic de-esterification of 3-acetoxindole with porcine liver esterase (Barnett & Seligman 1951), and tetrahydroborate reduction of indole-2,3-dione (Usami et al. 2001), respectively.
3.2.1.a Products of indole oxidation

The electronic absorption spectrum ($\lambda_{\text{max}}$ 248, shoulder ~280 nm), retention time ($t_R$ 23.0 min), and $m/z$ of the [M+H]$^+$ ion (134) of product I were identical to those of commercial 2-oxoindole (keto-tautomer). The very minor enol-tautomer (Joule & Mills 2010), 2-hydroxyindole, was not detected. Product II exhibited an electronic spectrum ($\lambda_{\text{max}}$ 262 and 321 nm) similar to that of N-FK (Makino & Tsuboi 1959) and an [M+H]$^+$ ion of $m/z$ expected for a dioxygenated indole derivative. Incubation of II in HCl (50 mM acid, 40 °C, ~1 hr) produced a new [M+H]$^+$ ion ($m/z$ 122) with an absorption spectrum ($\lambda_{\text{max}}$, 229 and 364 nm) identical to that of o-aminobenzaldehyde (Albert & Yamamoto 1966). II was, therefore, identified as o-formylaminobenzaldehyde.

Product III was identified as indole-2,3-dione (also known as isatin) on the basis of an electronic absorption spectrum ($\lambda_{\text{max}}$, 242, 302, 420 nm), mass spectrum ([M+H]$^+$ ion $m/z$ 148), and HPLC retention time (18 min) that were identical to those of an appropriate standard. Similarly, IV was identified as 3-hydroxy-2-oxoindole based on the physical properties ($\lambda_{\text{max}}$ 254 nm, shoulder ~291 nm; [M+H]$^+$ $m/z$ ion 150, $t_R$ 14.5 min) that were identical to those of indole-2,3-dione following anaerobic reduction with sodium dithionite (Sumpter 1945).

Product V was identified as 3-oxoindole (also known as indoxyl) because its properties ($\lambda_{\text{max}}$ 257 nm, 382 nm, [M+H]$^+$ $m/z$ ion 134, $t_R$ = 20 min) were the same as those of a product generated by treatment of 3-acetoxyindole with porcine liver esterase (Barrnett & Seligman 1951). The characteristic fluorescence emission spectrum of 3-oxoindole (470 nm emission, 380 nm excitation) indicated that it was the primary fluorophore observed during indole oxidation by IDO1. 3-oxoindole, however, oxidizes spontaneously to a non-fluorescent product under aerobic conditions (Cotson & Holt 1958), so its detection by HPLC required analysis within minutes after the reaction was initiated (vide infra). 3-hydroxyindole, a minor species that is in tautomeric
equilibrium with 3-oxoindole (Gaywood & McNab 2010), was not separable under the elution conditions employed although this form, specifically the enolate-anion, is thought to be the reactive species with O₂ (Russell & Kaupp 1969).

Product VI had an electronic absorption spectrum similar to that of 3-oxoindole (λ_max, 264 and 380 nm), but its mass spectrum ([M+H]⁺ ion m/z 132) indicated a mass 2 amu lower than that expected for 3-oxoindole. Reaction of 3-oxoindole with O₂ yielded two major products, one of which exhibited the same retention time (14.2 min) and absorption spectrum as VI, whereas the other product exhibited the same physical properties as III (indole-2,3-dione). In addition to the lower m/z, the observation of an absorbance band at 264 nm instead of 257 nm was consistent with a more conjugated system for VI relative to 3-oxoindole, suggesting that VI was 3-oxoindolenine, which has been reported to form by the reaction of 3-oxoindole with O₂ (Russell & Kaupp 1969). Finally, the mass spectrum of VII ([M+H]⁺ ion m/z 297) suggested that it formed by the dimerization of dioxygenated indole derivatives (i.e. bisindole derivative), but structural characterization of this product was not pursued.

As noted earlier, a blue, water and methanol insoluble product was obtained late in the course of indole oxidation by IDO1Fe³⁺ and H₂O₂. Analysis of this material by TLC revealed a single blue spot that co-migrated with commercial indigo blue (R₉ 0.33 in CHCl₃). The visible spectrum of this product (λ_max, 620 nm; shoulder, ~573 nm in DMSO) was also identical to that of the indigo blue standard. The identification of 3-oxoindole (V), the precursor of indigo blue (Cotson & Holt 1958, Russell & Kaupp 1969), further supports identification of this insoluble blue product as indigo blue. In some cases, traces of a pink pigment could be detected by TLC. This material likely corresponded to indirubin, which reportedly forms by the reaction of 3-oxoindole with indole-2,3-dione (McClay et al. 2005), both of which were found in the mixture of indole oxidation products. Indirubin presumably formed preferentially at high
concentrations of indole-2,3-dione, but the reaction conditions leading to its formation were not fully explored.

The physicochemical properties and electronic absorption spectra of the indole oxidation products are summarized in Table 3.1 and Figure 3.14.

3.2.1.b Products of 3-methylindole oxidation

Select methyl-substituted indoles were also readily oxidized by IDO1Fe$^{3+}$ and H$_2$O$_2$, and their oxidation products were characterized by electronic absorption spectroscopy and ESI-MS. At least three products (A1, A2, A3) were detected in the aerobic reaction of 3-methylindole with IDO1 and H$_2$O$_2$ (Figure 3.15 A). The ESI-MS analysis and the electronic absorption spectrum of A1 ([M+H]$^+$ m/z 164; $\lambda_{\text{max}}$ 229, 259, and 318 nm) were consistent with those previously reported for o-formylaminoacetophenone (Pileni et al. 1976). Spectroscopic changes resulting from incubation of A1 in 0.2 M HCl indicated deformylation to o-aminoacetophenone and further supported the assignment of A1 as o-formylaminoacetophenone. A2 was identified as 3-methyl-2-oxoindole on the basis of properties matching those of the commercial standard ([M+H]$^+$ m/z 148; $\lambda_{\text{max}}$ 250 nm, sh ~280 nm; $t_R$ 19.5 min). A3 appeared to be a bisindole derivative, as judged from its mass spectra ([M+H]$^+$ m/z 279). This product was likely formed through the dimerization of 3-methylindole and 3-methyl-2-oxoindole and in turn suggested the involvement of radicals. Further structural characterizations of A3, however, were not pursued. It is worth noting that in contrast to the oxidation of indole, oxidation of 3-methylindole proceeded without the detectable fluorescence emission at 470 nm (excitation 380 nm) characteristic of oxygen insertion at C$_3$. With the exception of o-formylaminoacetophenone, which involved ring-opening, no products were obtained in which oxygen was incorporated at the C$_3$ position of the indole ring. The methyl group at the C$_3$ position does not, in isolation, prevent oxygen insertion from occurring at this position, as oxygenated species of 3-methylindole at C$_3$.
Figure 3.14 Electronic absorption spectra of indole oxidation products. The spectra were recorded in 50% MeOH/H₂O (v/v), except for the compound II* and the MeOH insoluble pigment, which were recorded in 50 mM HCl and in DMSO, respectively. Label assignments are listed in Table 3.1. Numbers in parentheses denote the positions of absorption maxima.
Figure 3.15 Products of IDO1Fe$^{3+}$-catalyzed oxidation of 3-methylindole, 2-methylindole, and 2,3-dimethylindole by H$_2$O$_2$. HPLC analysis (gradient II; see section 2.6.3) of aerobic reaction components of IDO1Fe$^{3+}$ (50 μM protein in 20 mM Tris-HCl pH 7.5, 25°C) and (A) 3-methylindole (1 mM), (B) 2-methylindole (1 mM), and (C) 2,3-dimethylindole (200 μM), following addition of H$_2$O$_2$ to a final concentration of 500 μM. Numbers above the peaks denote retention times. Assignments of A1–A3, B1–B4, and C1–C3 are listed in Table 3.1. The insets depict the absorption spectra of the products; numbers in parentheses denote the positions of absorption maxima.
Table 3.1 Physical properties of the products from the IDO1Fe$^{3+}$-catalyzed oxidation of indoles by H$_2$O$_2$ $^a$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HPLC component</th>
<th>Abs$_{max}$ (nm)</th>
<th>Retention Time $^{b} t_R$ (min)</th>
<th>[M+H]$^+$ $^{m/z}$</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td>248, 280, sh</td>
<td>23.1</td>
<td>134</td>
<td>2-oxoindole</td>
</tr>
<tr>
<td>$^c$II</td>
<td></td>
<td>262, 321</td>
<td>21.4</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>242, 302, 420</td>
<td>17.9</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Indole</td>
<td>254, 291, sh</td>
<td>14.7</td>
<td>151</td>
<td>3-hydroxy-2-oxoindole</td>
</tr>
<tr>
<td>$^d$V</td>
<td></td>
<td>257, 382</td>
<td>19.9</td>
<td>134</td>
<td>3-oxoindole</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>264, 380</td>
<td>14.2</td>
<td>132</td>
<td>3-oxoindolenine</td>
</tr>
<tr>
<td>VII</td>
<td></td>
<td>239, 274, sh, 331</td>
<td>26.8</td>
<td>297</td>
<td>$^e$ bisindole derivative</td>
</tr>
<tr>
<td>insoluble pigment</td>
<td></td>
<td>620, 573, sh</td>
<td>nd</td>
<td>nd</td>
<td>indigo blue</td>
</tr>
</tbody>
</table>

$^c$A1    | 229, 259, 318 | 17.5             | 164                            | $^o$-formylaminoacetophenone |
| 3-methylindole |                   |                   |                                 | 3-methyl-2-oxoindole |
| A2        | 250, 280, sh   | 19.5              | 148                            |
| A3        | nd             | 27.1              | 279                            | $^e$ bisindole derivative |
### Table 3.1 (Continued)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HPLC component</th>
<th>$\text{Abs}_{\text{max}}$ (nm)</th>
<th>Retention Time $t_R$ (min)</th>
<th>$[\text{M+H}]^+$ $m/z$</th>
<th>Assignment</th>
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</thead>
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<tr>
<td>2-methylindole</td>
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<td>17.6</td>
<td>148</td>
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<tr>
<td></td>
<td>B2</td>
<td>231, 263, 397</td>
<td>21.5</td>
<td>293</td>
<td>bisindole derivative</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>231, 263, 397</td>
<td>21.8</td>
<td>293</td>
<td>bisindole derivative</td>
</tr>
<tr>
<td></td>
<td>B4</td>
<td>nd</td>
<td>23.0</td>
<td>277</td>
<td>bisindole derivative</td>
</tr>
<tr>
<td>2,3-dimethylindole</td>
<td>C1</td>
<td>nd</td>
<td>17.9</td>
<td>162</td>
<td>monooxygenated derivative</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>227, 259, 322</td>
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<td>178</td>
<td><img src="image" alt="o-acetamidoacetophenone" /></td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>nd</td>
<td>24.9</td>
<td>289</td>
<td>bisindole derivative</td>
</tr>
<tr>
<td>Commercial standards</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2-oxoindole</td>
<td></td>
<td>248, 280, 397</td>
<td>23.2</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>indole-2,3-dione</td>
<td></td>
<td>242, 302, 420</td>
<td>17.7</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>3-hydroxy-2-oxoindole</td>
<td></td>
<td>254, 291, 397</td>
<td>14.8</td>
<td>149</td>
<td></td>
</tr>
<tr>
<td>3-oxoindole</td>
<td></td>
<td>257, 382</td>
<td>19.9</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>indigo blue</td>
<td></td>
<td>620, 573, 673</td>
<td>n/d</td>
<td>262</td>
<td></td>
</tr>
<tr>
<td>3-methyl-2-oxoindole</td>
<td></td>
<td>248, 280, 397</td>
<td>19.5</td>
<td>147</td>
<td></td>
</tr>
</tbody>
</table>

*Reaction conditions: IDO1 (100-200 µM), H$_2$O$_2$ (0.5-4 mM), with indole (2 mM), 3-methylindole (1 mM), 2-methylindole (1 mM), or 2,3-dimethylindole (200 µM) in 20 mM Tris-HCl, pH 7.5, 20 °C, containing 0–2% MeOH. Reactions were quenched after 60 s. nd, not determined.

H$_2$O/MeOH elution using gradient I for indole and gradient II for the methyl-substituted indoles.

Deformylate to $\alpha$-aminobenzaldehyde in 50 mM HCl at 40 °C.

Unstable in O$_2$. 475 nm emission upon excitation 380 nm.

Structural characterization omitted.
(3-hydroxy-3-methylindolenine), C₃ and C₂ (3-hydroxy-3-methyl-2-oxoindole), and C₃-methyl (indole-3-methanol) are documented products from reactions catalyzed by cytochrome P450 enzymes (Skiles & Yost 1996, Skordos et al. 1998). This result, therefore, suggested that the lack of oxygen at C₃ in the IDO1-catalyzed reactions may be related to specific reaction conditions or to steric constraints imposed by the methyl group at the C₃ position.

3.2.1.c Products of 2-methylindole oxidation

As with indole, oxidation of 2-methylindole by IDO1Fe³⁺ and H₂O₂ also proceeded with increasing fluorescence emission in the 470 nm region. At least 4 products (B₁, B₂, B₃, B₄) resulted from this reaction (Figure 3.15 B). Product B₁ exhibited intense fluorescence emission at 475 nm, characteristic of 3-oxoindole derivatives (Gehauf & Goldenson 1957). The electronic absorption spectrum of this product (λ_max 229, 265, 402 nm) was similar to that of 3-oxoindole although the bands were found at 8–20 nm longer wavelengths presumably due to the extended conjugation from the C₂-methyl. In conjunction with its mass spectrum ([M+H]⁺ m/z 148), B₁ was identified as 2-methyl-3-oxoindole. Products B₂, B₃, and B₄ were also derivatives of 3-oxoindole based on the similarity of their absorption spectra to that of 3-oxoindole, whereas the mass spectra of these products indicated a mass that was in the range of bisindoles. B₂ and B₃ exhibited identical absorption spectra but could be resolved by HPLC (near 1:1 peaks less than 0.4 minutes apart). These results suggested that B₂ and B₃ constitute a racemic mixture, likely of the (2R, 2’S) and (2R, 2’R) conformations of 2,2’-dimethyl-2,2’-bisindoxyl (B₂ and B₃; [M+H]⁺ m/z 293), which had been synthesized earlier by Hassner and Haddadin (Hassner & Haddadin 1963). It is of interest to note that the proposed structures of B₂ and B₃ are analogous to that of leuco (reduced) indigo blue, but aerobic oxidation to yield an unsaturated C₂–C₂’ bond is not possible in the case of the 2,2’-disubstituted species. As a result, the product of the aerobic reaction of the 2-methylindole with IDO1Fe³⁺ and H₂O₂ was distinctly
yellow instead of the blue or indigo colour observed in reactions with indole. B4 ([M+H]^+ m/z 277) is 16 amu smaller than either B2 or B3, which in turn suggests that B4 resulted from the coupling of 2-methylindole with 2-methyl-3-oxoindole, as supported by the persistence of an absorption band (~280 nm) that is characteristic of the indole chromophore. The C3−C2' coupled structure formulated by Witkop (Witkop & Patrick 1951) is the probable structure of B4, but additional information is required to confirm this assignment.

3.2.1.d Products of 2,3-dimethylindole oxidation

Reactions involving 2,3-dimethylindole as substrate yielded 3 products (C1, C2, C3, Figure 3.15 C). The electronic absorption spectrum of C1 (λ_max 227, 259, 322 nm) was identical to that reported for o-acetamidoacetophenone (Pileni et al. 1976). Consistent with this identification, ESI-MS analysis of C1 yielded the anticipated [M+H]^+ ion of 178 m/z. On the basis of their ESI-MS data, C2 and C3 appear to correspond to monooxygenated ([M+H]^+ m/z 162) and dimeric ([M+H]^+ m/z 289) species of 2,3-dimethylindole, respectively, although the position at which coupling and/or oxygen insertion occurred was not identified.

3.2.2 Stoichiometry of indole oxidation by IDO1Fe^{3+} and H_2O_2

The reaction initiated by addition of equimolar amounts of both substrates to IDO1Fe^{3+} was monitored fluorimetrically to determine the stoichiometry of the reaction (Figure 3.16 A). Under these conditions (substrates, 26–416 μM; [IDO1], 10 μM), indole was consumed nearly quantitatively (~95 %). This result is consistent with one-to-one consumption of the two co-substrates, although presumably some peroxide was consumed in downstream formation of 2,3-dioxygenated indole species. Addition of a second equivalent of H_2O_2 upon completion of reaction led to oxidation of the remaining indole confirming that H_2O_2 depletion accounted for premature termination of the reaction. At lower enzyme concentration (i.e., 1 μM), less indole was oxidized. Under these conditions, reactions could only be re-established by addition of fresh
Figure 3.16 Stoichiometry of the oxidation of indole by IDO1Fe$^{3+}$ and H$_2$O$_2$. (A) Loss of indole (440 nmol initially) by aerobic reaction with IDO1Fe$^{3+}$ (10 nmol in 20 mM Tris-HCl, pH 7.5) as monitored by fluorescence emission (345 nm emission, 280 nm excitation), following addition of 440 nmol of H$_2$O$_2$ at timepoints indicated by the arrows. The inset plots nmoles of indole oxidized against nmoles of H$_2$O$_2$ added to solutions containing 1 and 10 nmoles IDO1Fe$^{3+}$ (closed and open circles, respectively). (B) Stalling and restoration of the reaction of indole (422 nmol) with H$_2$O$_2$ (1 μmol) and multiple additions of IDO1Fe$^{3+}$ (0.5 nmol each) at time points indicated by the arrows.
enzyme; however, diminishing amounts of indole were oxidized following each addition (Figure 3.16 B). As noted earlier, rapid decay of the Soret band was evident with additions of even modest concentrations of H$_2$O$_2$ to the free enzyme, so the loss in activity observed at lower [IDO1] was attributed to oxidative damage of the enzyme by peroxide.

3.2.3 Dependence of product distribution on H$_2$O$_2$ concentration

Whereas most products formed in the reaction of indole with IDO1Fe$^{3+}$ and H$_2$O$_2$ could not be monitored by electronic spectroscopy in real-time without spectroscopic deconvolution, 3-oxoindole is unique in this regard because its large Stokes shift (~90 nm) facilitated direct detection relatively free of interference by fluorescence emission at 470 nm. In the presence of excess H$_2$O$_2$ to indole, the 470 nm fluorescence emission increased initially, but decayed with time presumably as the result of 3-oxoindole conversion to indigo blue by O$_2$. Increasing [IDO1] in this case, increased both the rates of 3-oxoindole formation and decay based on the faster rise and fall of the fluorescence and indicated that the enzyme-catalyzed oxidation was not limited to the starting indole substrate.

The distribution of indole oxidation products following reaction (1 min) at fixed [indole] but varying [H$_2$O$_2$] was analyzed by HPLC, and the product concentrations were determined from the peak areas in the chromatograms (Figure 3.17). With initial [indole] > [H$_2$O$_2$], the major products were 2- and 3-oxoindole in a ~3:1 ratio with formation of trace amounts of o-formylaminobenzaldehyde. The yield of each of these products increased with [H$_2$O$_2$], but formation of 3-oxoindole was maximal when [indole] ≈ [H$_2$O$_2$] while 2-oxoindole formed maximally when H$_2$O$_2$ is ~2-fold in excess to indole. On the other hand, 3-hydroxy-2-oxoindole and indole-2,3-dione were detected only when [H$_2$O$_2$] > [indole], and their yields increased with increasing excess of H$_2$O$_2$ as was observed also for VII although the latter product could not be quantified. The pattern of product accumulation suggested involvement of secondary
Figure 3.17 $\text{H}_2\text{O}_2$ concentration-dependence of the distribution of indole oxidation products. The reactant and product concentrations as determined by HPLC analysis after 60 s reaction of IDO1Fe$^{3+}$ and indole (200 µM protein and 2 mM indole in 20 mM Tris-HCl pH 7.5, 25°C) following addition of $\text{H}_2\text{O}_2$ to a final concentration of 0.5–6 mM, or equivalently, to a ratio of 0.25–3 [H$_2$O$_2$]/[indole]. Indole, ○; 2-oxoindole, ●; 3-oxoindole, □; o-formylamino-benzaldehyde, Δ; indole-2,3-dione, ♦; and 3-hydroxy-2-oxoindole, ▲.
oxidation reactions driven by the availability of excess of peroxide once the initial substrate, indole, had been depleted. Notably, however, as H₂O₂ concentration increased, the sum of known [product] was less than the initial [indole]. Bisindole type products, including VII and indigo blue, were not quantified in this analysis but it is presumed that they accounted for a substantial fraction of products formed at high [H₂O₂].

3.2.5 Oxidation of 3-oxoindole and 2-oxoindole by IDO1Fe³⁺ and H₂O₂

The IDO1Fe³⁺-catalyzed oxidations of 2- and 3-oxoindole by H₂O₂ were examined separately to determine whether one or both of these monooxygenated products are further oxidized to form the dioxygenated products. The aerobic reaction of IDO1 with HPLC-purified 2-oxoindole and H₂O₂ (1 min) yielded the anticipated dioxygenated products indole-2,3-dione and 3-hydroxy-2-oxoindole (Figure 3.18 A). However, in contrast to indole, 2-oxoindole reacted poorly, so conditions that could lead to quantitative oxidation of 2-oxoindole could not be identified. The basis for this observation remains unclear, but the poor reactivity of 2-oxoindole is consistent with the accumulation of 2-oxoindole as the main product during IDO1 catalysis of indole oxidation by H₂O₂.

3-Oxoindole was prepared in situ by de-esterification of 3-acetoxyindole with porcine liver esterase (Barnett & Seligman 1951), and all work with this preparation prior to HPLC analysis was performed anaerobically to prevent spontaneous oxidation of this substrate by O₂. Under anaerobic conditions, 3-oxoindole was stable for hours provided either H₂O₂ or IDO1Fe³⁺ were excluded from solution. In cases where only IDO1Fe³⁺ was present, some oxidation of 3-oxoindole did occur eventually, but the extent of this oxidation was limited by the amount of enzyme present. Once all of the protein had been reduced (see section 3.2.10), there was no other electron acceptor available to oxidize more 3-oxoindole. Similarly, 3-oxoindole oxidized eventually in the presence of H₂O₂ ostensibly due to the slow disproportionation of peroxide to O₂. In cases where of both IDO1Fe³⁺ and H₂O₂ were present, 3-oxoindole is rapidly oxidized
Figure 3.18 2-oxoindole and 3-oxoindole as intermediates of indole oxidation. (A) HPLC analyses of reaction components from aerobic incubation (60 s) of 2-oxoindole with H$_2$O$_2$ (2 mM each in 20 mM Tris-HCl pH 7.5, trace a), and with addition of IDO1Fe$^{3+}$ (100 µM; trace b). (B) Reaction components from anaerobic incubation (60 s) of 3-oxoindole with H$_2$O$_2$ (1 mM each in 20 mM Tris-HCl pH 7.5, trace x), and with addition of IDO1Fe$^{3+}$ (50 µM; trace y). Trace z is obtained following 2 hr aerobic incubation of 3-oxoindole with no additions. Peak assignments are listed in Table 3.1. The inset in panel (B) depicts the progress of anaerobic oxidation of 3-oxoindole (100 µM) as monitored by fluorescence emission at 470 nm (390 nm excitation) by i, IDO1Fe$^{3+}$ and H$_2$O$_2$ (2.5 µM and 100 µM, respectively, added in sequence at the times indicated by the arrows); and ii, H$_2$O$_2$ alone. iii, progress of aerobic incubation of 3-oxoindole with no additions.

judging from fluorescence emission changes at 470 nm and HPLC analysis (Figure 3.18 B). This reaction resulted in the production of 3-oxoindolenine and indigo blue but no other significant dioxygenated indole derivatives were produced. On the other hand, the reaction of 3-oxindole by O$_2$ (~2 h) in the absence of IDO1 did afford dioxygenated indole (indole-2,3-dione) as a by-product as previously reported (Cotson & Holt 1958), suggesting that at least two pathways
could account for this product during IDO1Fe$^{3+}$-catalyzed oxidation of indole by H$_2$O$_2$: (a) enzymatic oxidation of 2-oxoindole as above, and (b) non-enzymatic oxidation of 3-oxoindole by O$_2$.

Whereas o-formylaminobenzaldehyde and the unidentified product VII were detected when indole was the initial substrate, neither of these two compounds was produced in the oxidation of either 2-oxoindole or 3-oxoindole, suggesting that these products formed by pathways different from those resulting in indole-2,3-dione or 3-hydroxy-2-oxoindole.

3.2.4 Role of superoxide and hydroxyl radicals

Oxidation of L-Trp has been reported to occur following reaction with hydroxyl radical (OH$^\cdot$) (Maskos et al. 1992) or following reaction of L-Trp$^{++}$ with O$_2^{\cdot -}$ (Kazakov et al. 2010). Consequently, the possible role of these radicals in the oxidation of indole by IDO1 and H$_2$O$_2$ was investigated. Inclusion of SOD ($\leq$ 1 $\mu$g/µL) in the reaction mixture did not significantly affect the rate or extent of indole oxidation (Table 3.2), or the distribution of products generated during reaction (Figure 3.19 A). Similarly, hydroxyl radical and singlet oxygen are unlikely participants in the reaction because neither the product distribution nor the progress of the reaction was influenced by the radical scavengers D-mannitol or lidocaine (Figure 3.19 B) (Das & Misra 1992).

<table>
<thead>
<tr>
<th>Table 3.2 Effects of scavengers on the rates of indole oxidation by IDO1Fe$^{3+}$ and H$_2$O$_2$.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$v$ (µM indole/min)</td>
</tr>
<tr>
<td>Complete system (C.S.)$^a$</td>
</tr>
<tr>
<td>C.S. + SOD</td>
</tr>
<tr>
<td>C.S. + lidocaine</td>
</tr>
<tr>
<td>C.S. + D-mannitol</td>
</tr>
<tr>
<td>C.S. under N$_2$ + D-glucose + glucose oxidase</td>
</tr>
</tbody>
</table>

$^a$ Complete system: 2, 90, 200 µM IDO1, indole, and H$_2$O$_2$, respectively, in 20 mM Tris-HCl pH 7.5, 20°C, including as indicated: 1.4 unit/µL SOD, 400 µM lidocaine in 1% methanol, 2 mM D-mannitol, or 10 mM D-glucose and 24 unit/mL glucose oxidase in N$_2$-purged buffer.

$^b$ Determined by monitoring loss of indole fluorescence 345 nm emission, 280 nm excitation)
Figure 3.19 Products of IDO1Fe\(^{3+}\)-catalyzed oxidation of indole by H\(_2\)O\(_2\) in the presence of scavengers. Product distribution revealed by HPLC using conditions and methods essentially as described in Figure 3.13, with (A) no additions (trace a), with SOD (2.8 unit/µL, trace b), with (B) lidocaine (400 µM) in 2% MeOH, and with (C) D-glucose (50 mM), glucose oxidase (47 unit/mL), in N\(_2\) purged buffer.

3.2.6 \(^{18}\)O incorporation in oxidation products of indole

Oxidation of indole by H\(_2\)O\(_2\) as catalyzed by IDO1Fe\(^{3+}\) proceeded under anaerobic conditions and resulted in a distribution of oxidation products similar to those observed under aerobic conditions (Figure 3.19 C), although the relative amount of 3-oxoindole (retention time ~20 min) was increased presumably as a result of reduced exposure to O\(_2\). The rate of the reaction was also unchanged under anaerobic conditions, even with the inclusion of glucose oxidase and D-glucose, leaving H\(_2\)O\(_2\) and H\(_2\)O as potential sources of oxygen in this reaction. Consequently, oxidation of indole by IDO1Fe\(^{3+}\) was performed with H\(_2\)^{18}O\(_2\) to determine whether this oxidant is the main source of oxygen in the reaction (Table 3.3). ESI-MS analysis
Table 3.3 Incorporation of $^{18}$O from H$_2$O$_{18}$O in the IDO1Fe$^{3+}$-catalyzed oxidation of indole and methyl-substituted indoles

<table>
<thead>
<tr>
<th>Product</th>
<th>Oxidant</th>
<th>$^{a}$ [M+H]$^+$ ion relative abundance (%)</th>
<th>$^{b}$ $^{18}$O incorporated (%)</th>
<th>$^{18}$O</th>
<th>$^{16}$O$^{18}$O</th>
<th>$^{16}$O$^{18}$O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$m/z$ 134 135 136 137 138</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-oxoindole</td>
<td>H$_2^{16}$O$_2$</td>
<td>100  8.9  0.8  0  0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>8.3  1.8  100  8.4  0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  8.8  0.7  0  0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>10.6  4.2  100  9.5  0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-oxoindole</td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.9  1  0.1  0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>19.9(100) 4.4(10.8) 100(74.8) 10.4(7.3) 58.4(9.1) 3.8(2.2)</td>
<td></td>
<td>n/a</td>
<td>56[62] 32[36]</td>
<td></td>
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<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>6.9(8.9) 5.9(2.7) 100(100) 9.8(10.5) 45.4(35.1) 4.9(6.7)</td>
<td></td>
<td>n/a</td>
<td>65[72] 30[33]</td>
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<tr>
<td>o-formylamino-benzaldehyde</td>
<td>H$_2^{16}$O$_2$</td>
<td>100  8.7  1.21  1.0  0.3  0</td>
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<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>28.0(66.1) 4.4(9.4) 100(100) 13.1(10.4) 2.2(2.9) 0(2.3)</td>
<td></td>
<td>n/a</td>
<td>78[87] 0[0]</td>
<td></td>
</tr>
<tr>
<td>3-hydroxy-2-oxoindole</td>
<td>H$_2^{16}$O$_2$</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>8.4  0.7  100  9.8  0.9</td>
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<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.7  0.6  0.1  0</td>
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<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>33  5.5  100  10.5  1.0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Indole-2,3-dione</td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.9  0.7  0.1  0</td>
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<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>28.0(66.1) 4.4(9.4) 100(100) 13.1(10.4) 2.2(2.9) 0(2.3)</td>
<td></td>
<td>n/a</td>
<td>78[87] 0[0]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.9  0.7  0.1  0</td>
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<td></td>
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<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>8.4  0.7  100  9.8  0.9</td>
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<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.7  0.6  0.1  0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>33  5.5  100  10.5  1.0</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.9  0.7  0.1  0</td>
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<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>8.4  0.7  100  9.8  0.9</td>
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<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.7  0.6  0.1  0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>33  5.5  100  10.5  1.0</td>
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<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.9  0.7  0.1  0</td>
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</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>8.4  0.7  100  9.8  0.9</td>
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<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.7  0.6  0.1  0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>33  5.5  100  10.5  1.0</td>
<td></td>
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<tr>
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<td>H$_2^{16}$O$_2$</td>
<td>100  9.9  0.7  0.1  0</td>
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<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>8.4  0.7  100  9.8  0.9</td>
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<tr>
<td></td>
<td>H$_2^{16}$O$_2$</td>
<td>100  9.7  0.6  0.1  0</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H$_2^{18}$O$_2$</td>
<td>33  5.5  100  10.5  1.0</td>
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</table>
Table 3.3 (Continued)

<table>
<thead>
<tr>
<th>Product</th>
<th>Oxidant</th>
<th>$^a$ [M+H]$^+$ ion relative abundance (%)</th>
<th>$^b$ $^{18}$O incorporated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$m/z$ 164 165 166 167 168 169</td>
<td>$^{18}$O $^{16}$O$^{18}$O $^{18}$O$^{18}$O</td>
</tr>
<tr>
<td>$o$-formylamino-</td>
<td>$H_2^{16}$O$_2$</td>
<td>100 9.8 1.3 0.6 0.9 1.0</td>
<td>n/a 5[6] 3[3]</td>
</tr>
<tr>
<td>acetophenone</td>
<td>$H_2^{18}$O$_2$</td>
<td>100 13 6.7 4.9 3.1 8.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$m/z$ 178 179 180 181 182 183</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$o$-acetamido-</td>
<td>$H_2^{16}$O$_2$</td>
<td>100 11 1.4 0.3 0.2 0.7</td>
<td>n/a 0[0] 0[0]</td>
</tr>
<tr>
<td>acetophenone</td>
<td>$H_2^{18}$O$_2$</td>
<td>100 10.9 1.3 0.4 0.4 0.8</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Numbers in parentheses denote relative abundances from a repeat analysis following a 3hr incubation period.

$^b$ Numbers in brackets are normalized per the enrichment level of $H_2^{18}$O$_2$ used (~90%). n/a, not applicable.
of the 2-oxoindole and 3-oxoindole produced under aerobic conditions (\(^{16}\text{O}_2\)-saturated) revealed that both exhibit a new \([M+H]^+\) ion of \(m/z\) 136 in addition to the previously observed \([M+H]^+\) ion \((m/z\) 134) that was consistent with the incorporation of one atom of \(^{18}\text{O}\) into the monooxygenated products. Natural isotope abundance contributed slightly to the intensity of the +2 \(m/z\) ion and was corrected by the method outlined under Appendix D. The percent incorporation of \(^{18}\text{O}\) into 2- and 3-oxindole reflected the ~90 % enrichment level of H\(_2^{18}\text{O}_2\) used in these experiments. This result demonstrated unambiguously that the oxidation of indole by IDO1 and H\(_2\text{O}_2\) occurred with essentially stoichiometric insertion of oxygen from H\(_2\text{O}_2\) to indole to form the monooxygenated products. No loss of the \(^{18}\text{O}\) label in 2-oxoindole (i.e., through oxygen exchange with solvent) was evident after 3 h at room temperature while 3-oxindole oxidized spontaneously to indigo blue in this time.

ESI-MS analysis of the dioxygenated product \(o\)-formylaminobenzaldehyde obtained in the same aerobic reaction revealed two new \([M+H]^+\) ions \((m/z\) 154 and 152) in addition to trace amounts of the previously detected product ion \((m/z\) 150). In this case, the relative abundance of \(^{18}\text{O}\) in these ions corresponded to 56 and 32 % of singly- and doubly-\(^{18}\text{O}\)-labelled product, respectively. Over a ~3 hr period, \(^{16}\text{O}/^{18}\text{O}\) exchange was evident and resulted in nearly complete disappearance of the \(m/z\) 154 ion and the dominance of the fully \(^{16}\text{O}\)-labelled \(o\)-formylaminobenzaldehyde species. Both the lower than theoretical level of \(^{18}\text{O}\) incorporation in \(o\)-formylaminobenzaldehyde seen initially and the subsequent time-dependent \(^{16}\text{O}/^{18}\text{O}\) exchange were consistent with behavior reported for the related compound \(N\)-FK (Hayaishi et al. 1957) that undergoes oxygen exchange with H\(_2^{16}\text{O}\) (Ronsein et al. 2009).

Incorporation of \(^{18}\text{O}\) into 3-hydroxy-2-oxoindole and indole-2,3-dione was also evident. However, while at most one of the two oxygen atoms in indole-2,3-dione originated from H\(_2^{18}\text{O}_2\) (78 % incorporation of a single \(^{18}\text{O}\) atom), a second \(^{18}\text{O}\) could also be found in 3-hydroxy-2-oxoindole (65 and 30 % incorporation of one and two atoms of \(^{18}\text{O}\), respectively). Time-
dependent loss of the $^{18}$O label from both products was evident, but this loss was slower for 3-hydroxy-2-oxoindole than for indole-2,3-dione or $o$-formylaminobenzaldehyde, with just one oxygen atom (presumably the C$_3$ oxygen) exchanging with H$_2$O over a 3 hr period.

### 3.2.7 $^{18}$O incorporation in oxidation products of methylindoles

As with indole, IDO1-catalyzed oxidation of methyl-substituted indoles were also performed with H$_2^{18}$O$_2$ to examine the effects of C$_2$ and C$_3$ substituents on oxygen transfer (Table 3.3). The aerobic reaction of 3-methylindole and 2-methylindole with IDO1Fe$^{3+}$ and H$_2$O$_2$ proceeded with stoichiometric (92%) and partial incorporation (75%) of $^{18}$O from H$_2^{18}$O$_2$ into their respective monooxygenated products. In contrast, no $^{18}$O incorporation was evident in the monooxygenated derivative of 2,3-dimethylindole. Dioxygenated, pyrrole ring-opened products were also produced in the oxidation of 3-methylindole and 2,3-dimethylindole (i.e., $o$-formylaminoacetophenone and $o$-acetoaminoacetophenone, respectively), but the mechanism of their formation evidently differs from that for the conversion of indole to $o$-formylaminobenzaldehyde because neither product incorporated $^{18}$O from H$_2^{18}$O$_2$.

### 3.2.8 Kinetics of IDO1-catalyzed oxidation of indole

In general, the use of low [IDO1] (1 $\mu$M) in catalysis of indole oxidation by H$_2$O$_2$ resulted in ~100 turnovers during which the rate of the reaction declined hyperbolically. Inactivation of IDO1 under these conditions presumably results from the peroxidative damage to the protein that competes with its enzymatic activity. Therefore, the kinetic traces were fit qualitatively to a logarithmic function to estimate the initial rates of indole oxidation.

The rate of disappearance of indole as a function of [H$_2$O$_2$] in the IDO1Fe$^{3+}$-catalyzed reaction exhibited Michaelis-Menten behavior with $K_m$ ~1.1 mM for peroxide ([indole], 25–300 $\mu$M) (Figure 3.20 A, Table 3.4). Corresponding measurements as a function of [indole], on the other hand, exhibited biphasic kinetic behavior with increasing [substrate] (Figure 3.20 B).
Figure 3.20 Dependence of the rates of indole oxidation by IDO1Fe$^{3+}$ as a function of substrate and H$_2$O$_2$ concentrations. Initial rates ($v_{\text{ini}}$, s$^{-1}$) of the oxidation of indole by IDO1Fe$^{3+}$ (1 µM in 20 mM Tris-HCl, pH 7.5, 25°C) monitored by fluorescence (345 nm emission, 280 nm excitation) as a function of (A) indole concentrations at 500 µM H$_2$O$_2$, with no L-Trp (a), and with 2, 5, and 30 µM L-Trp (b, c, d, respectively), and (B) H$_2$O$_2$ concentrations at 300, 90, and 25 µM indole (i, ii, iii, respectively). Steady-state kinetic parameters were extracted by fitting of the data (A) and (B) to equation 3.2 (see section 3.2.8), and the classic Michealis-Menten equation, respectively.

Table 3.4 Indole oxidation kinetic parameters as catalyzed by IDO1Fe$^{3+}$ and H$_2$O$_2$ $^a$

<table>
<thead>
<tr>
<th>$[\text{H}_2\text{O}_2]$</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}1}$ (s$^{-1}$)</th>
<th>$K_{m1}$ (µM)</th>
<th>$k_{\text{cat}2}/K_{m2}$ (s$^{-1}$ µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[25] 2.6 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>1.27 ± 0.18</td>
<td>8.6 ± 3.8</td>
<td>1.6 × 10$^{-2}$</td>
<td></td>
</tr>
<tr>
<td>[90] 7.6 ± 0.7</td>
<td>1.3 ± 0.2</td>
<td>0.34 ± 0.07</td>
<td>3.1 ± 4.4</td>
<td>8 × 10$^{-3}$</td>
<td></td>
</tr>
<tr>
<td>[300] 19.7 ± 3.2</td>
<td>1.2 ± 0.4</td>
<td>nd</td>
<td>nd</td>
<td>8 × 10$^{-3}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Error estimates reported as standard error obtained by non-linear regression fitting of the data.

$^b$ Data obtained as a function of [H$_2$O$_2$] at concentrations of indole indicated by bracketed numbers (in µM).

$^c$ Data obtained as a function of [indole] at 500 µM H$_2$O$_2$. Numbers in brackets denote the inclusion of L-Trp to the indicated concentrations (in µM). Nd, values of $k_{\text{cat}1}$ and $K_{m1}$ are too small to obtain reliable estimates.

kinetic pattern resembled the behavior of several cytochrome P450-catalyzed reactions for which it has been suggested that the availability of a second substrate within the active site results in continuously increasing velocity at high [substrate] (Korzekwa et al. 1998, Hutzler et al. 2001, Atkins 2005). A multi-site kinetic model is also consistent with the conclusion that indole could bind to both IDO1Fe$^{3+}$ and the IDO1Fe$^{3+}$–Trp complex (Sono 1989). Therefore, the
concentration dependence of the initial rates was fitted to a single substrate, two-sites model (Korzekwa et al. 1998) according to equation 3.2 (assuming $K_{m2} >> K_{m1}$, see Appendix C).

$$v = \frac{V_{\text{max1}}[S] + \left(\frac{V_{\text{max2}}}{K_{m2}}\right)[S]^2}{K_{m1} + [S]}$$

(3.2)

where $K_{m1}$ and $V_{\text{max1}}$ represent the kinetic constants for the first site, and $K_{m2}$ and $V_{\text{max2}}$ are kinetic constants for the second site that operate following saturation of the first site. This analysis yielded an apparent $k_{\text{cat1}}$ of $1.3 \pm 0.2 \text{ s}^{-1}$, $K_{m1}$ of $9 \pm 4 \mu\text{M}$, and $k_{\text{cat2}}/K_{m2}$ of $1.6 \times 10^{-2} \text{ s}^{-1} \mu\text{M}^{-1}$ at 500 $\mu\text{M}$ H$_2$O$_2$. Saturation of the second site was not observed in the range of [indole] examined, but inclusion of as little as 5 $\mu\text{M}$ L-Trp strongly inhibited the reaction (Figure 3.20 B) and changed the kinetic traces from biphasic to monophasic as the result of near abolition of indole binding at site 1 ($k_{\text{cat1}} 0.05 \pm 0.02 \text{ s}^{-1}$), presumably owing to the higher affinity of L-Trp for the ferryl enzyme ($K_d 0.3 \mu\text{M}$, (Lu & Yeh 2011)). Nevertheless, concentrations of L-Trp as great as 30 $\mu\text{M}$ were insufficient to inhibit activity completely. Note that L-Trp, however, was not oxidized at any appreciable rate by IDO1Fe$^{3+}$ and H$_2$O$_2$ in the duration of these experiments.

### 3.2.9 Anaerobic formation of IDO1Fe$^{2+}$ by indole and H$_2$O$_2$

IDO1Fe$^{3+}$-catalyzed oxidation of indole was monitored anaerobically and with sub-stoichiometric [H$_2$O$_2$] (~0.8 molar equivalent relative to IDO1Fe$^{3+}$) to determine the oxidation state of IDO1 following turnover. The electronic spectrum of the enzyme under these conditions revealed that a minor fraction of IDO1Fe$^{3+}$ (~2 %) was reduced to the ferrous form within 30 s of adding the H$_2$O$_2$ and that reduction continued until ~12% of the protein was reduced (15 min) (Figure 3.21 A). An accompanying increase in the absorbance at 608 nm indicated that oxidized indigo blue accumulated during this time. At higher [H$_2$O$_2$] (5 molar equivalents),
Figure 3.21 Accumulation of IDO1Fe^{2+} through the anaerobic reaction of IDO1Fe^{3+} with indole and H_{2}O_{2}. (A) Changes in the electronic absorption spectrum during anaerobic reaction of IDO1Fe^{3+} with indole (5.8 µM protein, 200 µM indole in 20mM Tris-HCl, pH 7.5, 20°C) and 5 molar equivalents of H_{2}O_{2} (29 µM). Spectra were recorded prior to addition of H_{2}O_{2} addition (bold curve) and at intervals of 7.5 min thereafter. (B) IDO1Fe^{3+} (7 µM) in the presence of 3-acetoxyindole (70 µM), recorded prior to (initial bold curve) and following addition of porcine liver esterase (2 units/mL) to convert 3-acetoxyindole into 3-oxoindole, at 1 min intervals. The inset depicts indigo blue and IDO1Fe^{2+} accumulation as monitored at 608 nm (isosbestic point of IDO1Fe^{3+}→IDO1Fe^{2+} transition) and 559 nm (IDO1Fe^{3+} ε_{559}=5.23 mM^{-1}cm^{-1}, IDO1Fe^{2+} ε_{559}=15.03 mM^{-1}cm^{-1}), respectively.
IDO1Fe$^{2+}$ accumulation quadrupled (~50%). Immediately after addition of H$_2$O$_2$, [indole] based on the absorbance at 280 nm decreased briefly but stopped changing even though IDO1Fe$^{3+}$ reduction continued. Introduction of catalase after H$_2$O$_2$ addition to quench the reaction did not influence this reduction of the enzyme, arguing against the involvement of a coupled process in the turnover of indole and reduction of the ferric heme iron.

3.2.10 Anaerobic reduction of IDO1Fe$^{3+}$ by 3-oxoindole

While investigating the reaction of 3-oxoindole with IDO1Fe$^{3+}$, 3-oxoindole was found to oxidize to indigo blue (increasing absorbance at 608 nm) under anaerobic conditions in spite of the absence of H$_2$O$_2$. This result indicated that reduction of IDO1Fe$^{3+}$ is sufficient to permit formation of this product. In the absence of H$_2$O$_2$ and O$_2$, indigo blue formation led to nearly quantitative reduction of the enzyme to IDO1Fe$^{2+}$ ($\lambda_{max}$ 559 nm; isosbestic points 461.5 nm, 523 nm, Figure 3.21 B). This reaction proceeded much more slowly when the esterase was omitted from the reaction mixture, presumably reflecting the slow spontaneous de-esterification of 3-acetoxyindole. The esterase alone had no effect on either the oxidation state of the heme iron or on the 3-oxoindole that accumulated. No other product of indole oxidation was able to reduce IDO1Fe$^{3+}$, so the accumulation of IDO1Fe$^{2+}$ during anaerobic oxidation of indole by IDO1Fe$^{3+}$ as described in section 3.2.9 was attributed to the reduction of the enzyme by 3-oxoindole produced in the reaction.

3.2.11 IDO1-catalyzed indole oxidation as supported by organic peroxides

Peracetic and $m$-chloroperbenzoic acid ($m$-CPBA) also supported IDOF$^{3+}$-catalyzed oxidation of indole. The range of products generated by the peracids was similar to that generated with H$_2$O$_2$ (Figure 3.22). Under conditions where peracid was in excess to indole, indole was quantitatively oxidized within ~30 s, and indole-2,3-dione (the most highly oxidized indole product) accumulation was increased compared to equivalent reactions performed with
H₂O₂. In the absence of protein, peracids slowly oxidized indole under aerobic conditions, as reported previously (Witkop 1947, Witkop & Fiedker 1947). In this case, incubation of indole with peracetic acid and \( m \)-CPBA (2 mM and 4 mM, respectively) for 1 hr afforded \( o \)-formylaminobenzaldehyde as the primary product in addition to traces of 2-oxoindole. Formation of additional dimeric and trimeric indole species by peracids is reportedly possible (Witkop 1947, Witkop & Fiedker 1947) but was not detected under the reaction conditions employed.

Reaction of a highly-purified thermophilic cytochrome P450 with \( m \)-CPBA was recently shown to produce a relatively clean spectrum of the highly sought-after Compound I intermediate (Rittle & Green 2010). IDO1Fe³⁺ reacted with \( m \)-CPBA to form a species that exhibited the same spectroscopic features to that generated by reaction with H₂O₂, which were assigned to the ferryl heme only (IDO1Fe⁴⁺=O, (Lu & Yeh 2011)). As was the case with H₂O₂, formation of IDO1Fe⁴⁺=O occurred with a single set of isosbestic points; spectroscopic features characteristic of a porphyrin cation radical like Compound I of horseradish peroxidase or cytochromes P450 were not detected under these conditions, indicating that reduction and/or dissipation of such radical via the peptide chain occurred rapidly.

On the other hand, if peroxide bound to the heme iron dissociates via homolytic cleavage of the O–O bond, the initial enzyme product is anticipated to be the ferryl heme and not the porphyrin/protein cation radical. Homolytic cleavage of the O–O bond of cumene hydroperoxide reportedly yields cumyloxy radical, which spontaneously decarboxylates to yield acetophenone (Barr et al. 1996, Matsui et al. 1999, Yoshioka et al. 2000). In contrast, heterolytic cleavage yields the corresponding alcohol. The nature of O–O bond cleavage of the peroxide upon reaction with the heme iron of IDO1 is, therefore, reflected in the ratio of cumene alcohol to acetophenone. The reaction (15 min) of cumene hydroperoxide with IDO1Fe³⁺ resulted in quantitative conversion of the peroxide to cumene alcohol and acetophenone in a ratio of 3.2 to 1.
as determined by HPLC. The same reaction carried out using ferric myoglobin (sperm whale) produced a similar ratio of 3.3 to 1, in close agreement with the results obtained by Matsui and co-workers (Matsui et al. 1999). These observations indicated that cumene hydroperoxide dissociated from IDO1Fe$^{3+}$ predominantly by heterolysis of the O–O bond, presumably resulting in two-electron oxidation of the ferric heme.

![Figure 3.22](image)

**Figure 3.22** IDO1Fe$^{3+}$-catalyzed aerobic oxidation of indole with organic hydroperoxides. HPLC analysis (gradient II, see section 2.6.3) of aerobic reaction products of indole oxidation (2 mM in 200 mM potassium phosphate, pH 7.5) following 1 hr incubation with peracetic acid (*a*), and m-chloroperbenzoic acid (*m*-CPBA, *b*), to a final concentration of 2 and 4 mM, respectively; or following 30 s incubation with IDO1Fe$^{3+}$ (100 μM protein in 200 mM potassium phosphate pH 7.5) and 4 mM peracetic acid (*c*), and *m*-CPBA (*d*).

### 3.3 Characterization of indoleamine 2,3-dioxygenase 2 (IDO2)

#### 3.3.1 Homology modeling of the active-site of IDO2

Although the existence of IDO2 was not recognized until relatively recently, the high sequence identity with IDO1 and the fact that the two enzymes can catalyze the same reaction(s)
justified using the crystal structure of IDO1 as framework on which to model the three-dimensional structure of IDO2. Following the protocol described in section 2.7.2, the best model (Figure 3.23) exhibited a minimized root-mean-square deviation (RMSD) for equivalent $\alpha$-carbons (excluding the missing coordinates of residues 1–11 and 361–379 of IDO1) of 0.35 Å.

In agreement with the sequence alignment from which it was predicted that His360 provides the axial ligand to the heme iron on the proximal side, this coordination in the IDO2 model exhibits structural characteristics that are similar to that of IDO1. Specifically, Arg357, Asp291 and the 6-propionate of the heme form a hydrogen-bonding network on the proximal side of the heme in this IDO2 model. A cavity is predicted to form on this side between Asp291 and His360, which can conceivably accommodate water molecule(s) that correspond to those that bridge the interaction of His360 to Asp291. On the other hand, a pair of phenylalanyl residues that form stacking interactions with the heme in IDO1 (Phe214 and Phe270) are replaced by leucyl residues in IDO2 (Leu231 and Leu287). Similarly, Ile217 and Ile329 are replaced by Met234 and Met363, respectively, but their distances from the heme iron (7.2–7.5 Å from sulfur to iron atom) were too great for them to be considered as axial ligands without invoking significant conformational re-arrangement.

On the distal side of the heme, the polar residues closest to the heme include His143, Thr184, and Ser280 (topologically analogous to Tyr126, Ser167, and Ser263 of IDO1, respectively) (Figure 3.23 B). Ser280 interacts directly with the heme 7-propionate, whereas His143 and Thr184 do not appear to engage the heme; at 6.2 Å, the N$\varepsilon_2$ of His143 is not sufficiently close to the heme iron to act as a ligand directly without rearrangement of the cavity. The substrate binding pocket also resembles that of IDO1. Specifically, putative $\pi-\pi$ stacking interactions with the indole ring of L-Trp are preserved via the aromatic rings of Phe180 and Phe243 in IDO2. Other residues that may also participate in substrate binding include Arg248 at the entry to the distal pocket. Arg248 presumably interacts with the carboxylate of L-Trp, and is
Figure 3.23 Structural comparison of the active-sites of IDO1 and IDO2. The proximal (left) and distal (right) heme pocket of IDO2 (top) and IDO1 (bottom) and the surrounding residues viewed along the heme plane, with surface representations of the cavities which hold water molecules (red spheres in IDO1) that are involved in the proximal hydrogen bonding network. 4-Phenylimidazole complexed to IDO1 is shown in magenta. The structure of IDO2 was modeled with the program SwissPDB using the crystal structure coordinates of IDO1 (1D0T) as a framework.

maintained in position by means of cation-π interactions with Tyr244. The residues comprising the flexible distal loop (residue 250–267 and 267–284 in IDO1 and IDO2, respectively), appears largely conserved as well, particularly with respect to the postulated hinge region formed by the Ser- Gly-Gly-Ser-Ala motif, which is highly conserved among vertebrate IDOs. Finally no intramolecular disulfide bridges were predicted for the IDO2 structure.
3.3.2 The electronic structure of IDO2 and Δ27IDO2

The electronic spectrum of ferric IDO2 with the truncated N-terminus (Δ27IDO2Fe\(^{3+}\)) exhibited maxima (404, 502, and 632 nm) at pH 7.5 that are characteristic of a high-spin ferric heme, similar to that of IDO1 (Figure 3.24). The extinction coefficients of the Soret and the maxima at 280 nm for ferric protein were 178,500 M\(^{-1}\) cm\(^{-1}\) and 81,000 M\(^{-1}\) cm\(^{-1}\), respectively, based on pyridine hemochromagen assays. The average intensity of the Q-band region relative to that of the Soret band was lower for Δ27IDO2Fe\(^{3+}\) than for the spectrum of IDO1Fe\(^{3+}\). The well-resolved β-band at 502 nm and diffuse α-band at ~530 nm, indicated that there was no substantial population of low-spin heme although it was clearly greater in the full-length (wild-type) IDO2Fe\(^{3+}\) (red-shifted Soret at 404.5 nm, and better resolution in the α- and β-bands at ~540 and ~570 nm, respectively). However, aggregation of the wild-type protein was evident from the onset of light scattering, which advanced over the period of ~1 hr at 20°C. In contrast, the Δ27IDO2Fe\(^{3+}\) variant was stable spectroscopically under the same conditions for periods in excess of 24 hr, although it too deteriorated rapidly at temperatures ≥ 30°C. Because of this comparatively greater stability, the variant was used in all subsequent spectroscopic and functional studies.

As previously reported, addition of L-Trp to IDO1Fe\(^{3+}\) induced a red-shift of the Soret and β-band to 411 nm and 544 nm, respectively, and the appearance of the α-band as a shoulder around 575 nm. These changes presumably reflect the shift in equilibrium from His/H\(_2\)O to His/OH\(^-\) axial coordination upon L-Trp binding (Terentis et al. 2002, Davydov et al. 2010). Titration of L-Trp to Δ27IDOFe\(^{3+}\) resulted in similar changes as described above in the electronic spectrum of this enzyme (Figure 3.27) although these changes were comparably subdued in Δ27IDO2Fe\(^{3+}\) than in IDO1Fe\(^{3+}\) for the same [L-Trp]. The absorbance changes for Δ27IDO2Fe\(^{3+}\) were small at pH 7.5 even at high [L-Trp] (i.e., 15 mM) but they became significantly more
Figure 3.24 Comparison of the electronic absorption spectra of IDO2Fe³⁺, Δ27IDO2Fe³⁺, and IDO1Fe³⁺. Electronic absorption spectra of 3 μM (a) Δ27IDO2Fe³⁺, (b) IDO1Fe³⁺, and (c) wild-type IDO2Fe³⁺ recorded in 200 mM potassium phosphate buffer, pH 7.5, 20 °C. Numbers above the bands denote positions of the local absorption maxima (IDO1Fe³⁺ in parentheses). The inset depicts the progression of light scattering of Δ27IDO2Fe³⁺ monitored by absorbance changes at 280 nm upon increasing the temperature to 36°C as the timepoint indicated.

pronounced at pH 8.5. The progression of absorbance decreases at 404 nm as a function of [L-Trp] indicated that a substantial fraction of the enzyme remained as Δ27IDO2Fe³⁺ with Δ27IDO2Fe³⁺–L-Trp accounting for maximally ~66% even at [L-Trp] in excess of 15 mM (pH 8.5). The spectrum of Δ27IDO2Fe³⁺–L-Trp was simulated by the linear combination of the abstract spectra obtained from singular value deconvolution (SVD) analysis (using two factors).
Figure 3.25 L-Trp binding to Δ27IDO2Fe$^{3+}$. Changes in the electronic absorption spectra of Δ27IDO2Fe$^{3+}$ (5.1 µM protein in 20 mM Tris-HCl, pH 7.5 or pH 8.5, as indicated), following addition L-Trp the final concentrations 0.6 to 15.2 mM, as indicated. The spectrum of L-Trp-saturated Δ27IDO2Fe$^{3+}$ (bold) was extrapolated by the linear combination of the abstract spectra obtained from singular value deconvolution analysis of this set of spectra. Numbers in parentheses denote the positions of the absorption maxima. Inset depicts the fraction of free Δ27IDO2Fe$^{3+}$ as a function of L-Trp concentrations, which was determined from the change in absorbance at 404 nm. Fitting of this data to the Hill equation yields $K_d$ values of 7.4 ± 2.1 mM for the binding of L-Trp to Δ27IDO2Fe$^{3+}$ at pH 8.5.

of the series of spectra recorded for the titration. The predicted maxima of Δ27IDO2Fe$^{3+}$–L-Trp by this analysis were 412, 540, and shoulder at ~575 nm, which are similar to those of IDO1Fe$^{3+}$–L-Trp. The corresponding equilibrium dissociation constant ($K_d$) for L-Trp binding to Δ27IDO2Fe$^{3+}$ was estimated to be 7.4 ± 2.1 mM at pH 8.5 (Figure 3.25 inset), a value
substantially greater than that estimated for IDO1Fe\(^{3+}\) \((K_d 281 \pm 31 \mu M \text{ at pH 8.0, this study}; 285 \pm 6 \mu M \text{ at pH 8.0, (Chauhan et al. 2008); 0.9 mM at pH 7.0, (Lu et al. 2010)})\). Although all spectra nearly intersect at 412, 482, 518, and 602 nm suggesting that L-Trp binding to \(\Delta 27\text{IDO2Fe}^{3+}\) is a two-state transition, the fact that these intersection points are not perfect isosbestic points indicates the presence of minor third component.

In control studies where \(\Delta 27\text{IDO2Fe}^{3+}\) was incubated in Tris-HCl buffer (pH 8.5, 20 °C) in the absence of other ligands, the \(\alpha\)- and \(\beta\)-bands of \(\Delta 27\text{IDOFe}^{3+}\) also increased in intensity in the period of \(~3.5\) hr, concomitant with red-shift and weakening of the intensity of the Soret band indicating a change in the spin-state equilibrium towards the low-spin Fe\(^{3+}\) heme (Figure 3.26 A). This transition occurred with well-defined isosbestic points at 414.5, 485, 518, 602.5, and 654.5 nm. The spectrum of this low-spin \(\Delta 27\text{IDO2Fe}^{3+}\) was simulated by SVD analysis (using two factors). The \(\alpha\)- and \(\beta\)-bands of the predicted spectrum (542 and 577 nm, respectively) were similar in position and relative intensity to those of L-Trp-bound enzyme as described above. On the other hand, the Soret band (420 nm) was notably lower in energy than that of the L-Trp bound protein (412 nm) and was instead similar to the cyanide-bound protein (418 nm, vide infra). In comparison, a very small, consistent increase in the intensities of the \(\alpha\)- and \(\beta\)-bands of IDO1Fe\(^{3+}\) was resolved at pH 9.0 (Figure 3.26 B), but no further absorbance changes occurred with time. In the case of \(\Delta 27\text{IDO2Fe}^{3+}\), changes in the spin-state equilibrium occur very slowly and may be associated with formation of soluble protein dimers and/or oligomers. This suggestion is reasonable insofar as pH greater than 8.5 led to increasing light scattering in the spectrum of \(\Delta 27\text{IDO2Fe}^{3+}\) and eventually precipitation of the protein. This low-spin form of the enzyme is likely present as a minor component during titration of \(\Delta 27\text{IDO2Fe}^{3+}\) with L-Trp at pH 8.5, and may account for \(~5\)% of the decrease in the intensity of the Soret maximum (based on an estimated 20 minutes elapsed during titration).
Figure 3.26 Electronic absorption spectra of Δ27IDO2Fe³⁺ at mildly alkaline pH. (A) Changes in the spectra of Δ27IDO2Fe³⁺ (10 µM protein in 20 mM Tris-HCl, pH 8.5, 20°C) over a period of ~4 hrs, and replotted as changes in absorbance at 404nm (inset). The spectrum of the low-spin Δ27IDO2Fe³⁺ (dotted) was extrapolated by the linear combination of the abstract spectra obtained from singular value deconvolution analysis of this set of spectra. (B) Spectra of IDO1Fe³⁺ at pH 7.5 (in 20 mM Tris-HCl, solid) and pH 9.5 (in 20 mM TAPS, dashed). These spectra were stable over a period in excess of 2 hr.
In the presence of cyanide (CN\(^-\)), the Soret maximum, \(\alpha\)- and \(\beta\)-bands of \(\Delta 27\text{IDO2Fe}^{3+}\) were shifted to 418, ~568, and 538 nm, respectively (Figure 3.27 A), which is consistent with binding of CN\(^-\) at the heme iron (i.e., \(\Delta 27\text{IDO2Fe}^{3+}\text{--CN}^-\)). The \(\Delta 27\text{IDO2Fe}^{3+}\text{--CN}^-\) derivative (saturated at 6 \(\mu\)M CN\(^-\)) exhibited significantly greater affinity for L-Trp than did the ferric enzyme alone. In this case, the Soret maximum of \(\Delta 27\text{IDO2Fe}^{3+}\text{--CN}^-\) was red-shifted (from 418 to 421 nm, isosbestic point 420 nm) on titration with L-Trp while the \(\alpha\)- and \(\beta\)-bands showed very small changes (Figure 3.27 B). In contrast, titration of \(\text{IDO1Fe}^{3+}\text{--CN}^-\) with L-Trp shifted the Soret maximum to higher energy (from 418 to 416 nm, isosbestic point 441 nm, Figure 3.27 C). The \(K_d\) estimated from spectroscopic titrations of this form of \(\Delta 27\text{IDO2}\) with L-Trp was 90 ± 15 \(\mu\)M at pH 7.5, representing at least a 80-fold enhancement in affinity for L-Trp compared to \(\Delta 27\text{IDO2Fe}^{3+}\), but approximately 7-fold lower affinity than \(\text{IDO1Fe}^{3+}\text{--CN}^-\) (13 ± 3 \(\mu\)M at pH 7.5, this study; 18 ± 3 \(\mu\)M at pH 7.4, (Lu et al. 2010)).

The electronic spectrum of \(\Delta 27\text{IDO2Fe}^{2+}\) exhibited a single broad band centered at 557 nm that is consistent with a high-spin penta-coordinate heme (Figure 3.28) similar to that observed for IDO1. No substantial pH-dependent absorbance changes were detected for \(\Delta 27\text{IDO2Fe}^{2+}\). Binding of O\(_2\) to the ferrous protein resulted in the characteristic change to low-spin heme, evident from the red-shifted Soret (412 nm) and well-resolved \(\alpha\)- and \(\beta\)-bands at 576 and 541 nm, respectively (Figure 3.28). In both cases, the near complete disappearance of the charge transfer band (631 nm) indicated the near absence of \(\text{IDOFe}^{3+}\) initially although spontaneous oxidation of \(\Delta 27\text{IDO2Fe}^{3+}\text{--O}_2\cdot^-\) to the ferric state occurred with time (see section 3.3.5 for kinetic characterization).

### 3.3.3 L-Trp dioxygenase activity of \(\Delta 27\text{IDO2}\)

\(\Delta 27\text{IDO2}\) was less efficient at oxidizing L-Trp than is IDO1. Values of \(k_{cat}\) and \(K_m\) obtained with the standard steady-state assay conditions (i.e., chemical reduction by methylene
Figure 3.27 L-Trp binding to Δ27IDO2Fe³⁺–CN⁻. (A) The electronic absorption spectra of Δ27IDO2Fe³⁺ (3.7 µM in 20 mM Tris-HCl, pH 7.5) following titration of cyanide to a final concentrations 6 µM. Changes in the spectra of cyanide saturated (B) Δ27IDO2Fe³⁺ and (C) IDO1Fe³⁺ upon titration with L-Trp (0.1–7.3 mM and 3–430 µM, respectively). Insets depict changes in absorbance at 418 nm as a function of L-Trp concentrations. Fitting of this data to the Hill equation yields $K_d$ values of 90 ± 15 and 13 ± 3 µM for the binding of L-Trp to Δ27IDO2Fe³⁺–CN⁻ and IDO1Fe³⁺–CN⁻, respectively, at pH 7.5. Number in parenthesis denotes isosbestic point.
Figure 3.28 Electronic absorption spectra of Δ27IDO2Fe^{2+} and Δ27IDO2Fe^{3+}−O_2^{•−}. Δ27IDO2 (5 µM protein in 20 mM Tris-HCl, pH 7.5, 20°C) in the ferric (Fe^{3+}, dotted), ferrous (Fe^{2+}, solid), and dioxygen-bound (Fe^{3+}−O_2^{•−}, bold) states. The isosbestic points of the Fe^{3+}→Fe^{2+} transition were 415, 459, 521, and 605 nm. The isosbestic points of the Fe^{2+}→Fe^{3+}−O_2^{•−} transition were 411, 471, 524, and 593 nm.

blue and ascorbate) were 11.0 ± 0.3 min⁻¹ and 3.2 ± 0.3 mM, respectively, at pH 7.5 and 20 °C (Figure 3.29 A). The apparent specificity constant (k_{cat}/K_m) was ~2,500-fold lower (3.2 ± 0.3 min⁻¹ mM⁻¹) than the corresponding value for IDO1 (k_{cat}/K_m 8.1 ± 0.9 × 10³ min⁻¹ mM⁻¹ at pH 6.5 and 20 °C). These parameters are comparable to those of murine IDO2, which also exhibits diminished activity relative to its isoform (Austin et al. 2010, Yuasa et al. 2010). HPLC analysis of the reaction products revealed no significant oxidation products other than N-FK and its
decomposition product, kynurenine. D-Trp, 5-OH-L-Trp and tryptamine were similarly poor substrate for this enzyme.

As a result of the relatively high reduction potential of its Fe$^{3+}$/Fe$^{2+}$ couple (see section 3.3.4), Δ27IDO2 (and IDO1) activity can be activated with conventional protein mediators, including cytochromes $b_5$ (bovine microsomal; $E_m +5$ mV, (Funk et al. 1990)), and ferredoxins (spinach; $E_m -420$ mV, (Cammack et al. 1977)). In each case, reduction proceeded anaerobically and resulted in families of spectra with isosbestic points similar to those obtained by chemical and photochemical reduction. A steady-state activity assay was developed that used cytochrome $b_5$ and cytochrome $b_5$ reductase as the reducing system. This cytochrome has been proposed to be the physiological reductant of IDO1 (Vottero et al. 2006, Maghzal et al. 2008), and probably, IDO2 as well. Using this system (10 µM cytochrome $b_5$, 150 nM cytochrome $b_5$ reductase, and 150 µM β-NADH in 20 mM Tris-HCl pH 7.5), the $K_m$ for Δ27IDO2 was approximately 7-fold lower (410 ± 20 µM) than that obtained by the methylene blue and ascorbate system, whereas $k_{cat}$ was unchanged (10.1 ± 0.1 min$^{-1}$) (Figure 3.29 B). These results are consistent with those reported for murine IDO2, which also exhibits an increase in the apparent affinity for L-Trp when using a coupled reduction system based on cytochrome $b_5$ to activate the protein (12 mM to 530 µM, (Austin et al. 2010)). Interestingly, although the the L-Trp dioxygenase activity of IDO1 is inhibited at high [L-Trp] ($K_{si}$ 740 ± 80 µM at pH 6.5, this study; 170 µM at pH 7.5, (Lu et al. 2009)), no apparent substrate inhibition was observed in Δ27IDO2 with [L-Trp] up to 5 and 15 mM in assays employing the enzymatic and chemical reducing systems (pH 7.5), respectively.

3.3.4 The Fe$^{2+}$/Fe$^{3+}$ midpoint reduction potential of Δ27IDO2

The midpoint reduction potential ($E_m$) of the Δ27IDO2 Fe$^{3+}$/Fe$^{2+}$ couple as measured by the photochemical reduction method (see section 2.7.4) was $-34.8 \pm 1.3$ mV versus SHE
Figure 3.29 Dependence of the rates of L-Trp oxidation by Δ27IDO2 as a function of substrate concentration.

Initial rates ($v_{\text{ini}}$, min$^{-1}$) of the conversion of L-Trp to N-FK by Δ27IDO2 (solid curve) monitored by the increasing absorbance at 321 nm using (A) chemical reducing system comprised of ascorbic acid, methylene blue, and bovine liver catalase (20 µM, 20 mM, and 50 µg/mL, respectively) in 100 mM potassium phosphate buffer, pH 7.5, or (B) enzymatic reducing system comprised of cytochrome $b_5$, cytochrome $b_5$ reductase, and β-NADH (10 µM, 150 nM, 150 µM, respectively) in 20 mM Tris-HCl buffer, pH 7.5. Corresponding rates for the reaction catalyzed by IDO1 using the same chemical reducing system in 100 mM potassium phosphate buffer, pH 6.5 (panel A, dashed curve). All reactions were performed at 20 °C. The Michaelis-Menten parameters were obtained by non-linear regression fitting of the data and listed in Table 3.5.

Table 3.5 Kinetic parameters of the L-Trp dioxygenase activity of Δ27IDO2

<table>
<thead>
<tr>
<th>System</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}/K_m$ (min$^{-1}$ mM$^{-1}$)</th>
<th>$K_s$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ27IDO2 + methylene blue/ascorbate</td>
<td>11.0 ± 0.3</td>
<td>3,200 ± 300</td>
<td>3.4 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>Δ27IDO2 + Cyt$b_5$/Cyt$b_5$-reductase</td>
<td>10.1 ± 0.1</td>
<td>410 ± 10</td>
<td>25 ± 1</td>
<td>nd</td>
</tr>
<tr>
<td>IDO1 + methylene blue/ascorbate</td>
<td>$b$ 111 ± 3</td>
<td>$b$14 ± 1</td>
<td>8,100 ± 900</td>
<td>$b$ 740 ± 80</td>
</tr>
</tbody>
</table>

$^a$ Error estimates reported as standard error obtained by non-linear regression fitting of the data. Nd, no substrate inhibition detected.

$^b$ Parameters obtained by fitting of the data to equation A10 (see Appendix C).
at pH 7.0 (Figure 3.30). Measurements carried out with IDO1Fe$^{3+}$ under the same conditions yielded a value of $-42.4 \pm 0.2$ mV, which is in reasonable agreement with previously reported values ($-30$ mV, (Papadopoulou et al. 2005) and $-63$ mV, (Chauhan et al. 2008)). The $E_m$ of methylene blue in solution decreases with increasing pH, and is $\sim -6$ mV at pH 8.0 (Pessoa et al. 1997). Based on this value, values of $E_m$ at pH 8.0 for $\Delta27$IDO2 and IDO1 were $-35.9 \pm 0.5$ and $-48.2 \pm 0.5$ mV, respectively, which were similar to their respective values obtained at pH 7.0. The slopes from the plots of the logarithms of oxidized/reduced enzyme (ordinate) versus oxidized/reduced dye (abscissa) in each case were approximately 0.5, consistent with one and two electron processes for $\Delta27$IDO2 ($n = 1$) and methylene blue ($n = 2$), respectively (Figure 3.30 B). The less negative $E_m$ of $\Delta27$IDO2 may be accounted for by a heme environment of lower dielectric constant (Kassner 1972) and suggests that the ferrous form of this protein is more stable than that of IDO1 although other factors (e.g., O$_2$ binding affinity and susceptibility of the $\Delta27$IDO2Fe$^{3+}$–O$_2$•− derivative to auto-oxidation) undoubtedly contribute to the overall catalytic activity of the enzyme. Notably, binding of L-Trp to IDO1Fe$^{3+}$ has been correlated with an increase in the $E_m$ (Efimov et al. 2012), but this effect could not be evaluated for $\Delta27$IDO2 owing to the low affinity of the ferric enzyme for L-Trp.

### 3.3.5 Auto-oxidation of $\Delta27$IDO2Fe$^{3+}$–O$_2$•− to $\Delta27$IDO2Fe$^{3+}$

Oxygenated $\Delta27$IDO2 ($\Delta27$IDO2Fe$^{3+}$–O$_2$•−) readily auto-oxidized to the ferric state. The inclusion of SOD and catalase made this process essentially irreversible. O$_2$ concentration was not controlled during data acquisition; initial [O$_2$] was $\sim 140$ µM (based on an estimated dissolved [O$_2$] of 280 µM at 20 °C for air-saturated buffer), but it probably increased during data acquisition as the solution equilibrated with the atmosphere. Nevertheless, the progress of decay of $\Delta27$IDO2Fe$^{3+}$–O$_2$•− as monitored at 521 nm, the isosbestic point for the ferric and ferrous
Figure 3.30 Fe$^{3+}$/Fe$^{2+}$ midpoint reduction potentials of Δ27IDO2 compared to those of IDO1 at pH 7 and 8.

(A) Representative series of spectra for the anaerobic photochemical titration of Δ27IDO2Fe$^{3+}$ (5 µM protein in 100 mM potassium phosphate pH 7–8 and 10 mM EDTA) with 10 µM methylene blue as reduction mediator and reporter. (B) Corresponding linear Nernst plots used in the determination of the Fe$^{3+}$/Fe$^{2+}$ midpoint potentials of Δ27IDO2 and IDO1, at pH 7 and 8, as indicated.
enzyme, conformed remarkably well to first-order reaction kinetic. Modeling of the progress curves by a mono-exponential function yielded an apparent rate constant of auto-oxidation \(K_{\text{ox}}\) of \(6.9 \pm 0.2 \times 10^{-2} \text{ min}^{-1}\) at pH 6.5 (Figure 3.31). Values of \(K_{\text{ox}}\) exhibited strong pH-dependence and indicated that the enzyme was more susceptible to auto-oxidation at acidic pH \(K_{\text{ox}} = 7.3 \pm 0.5 \times 10^{-3} \text{ min}^{-1}\) at pH 8.5). At the pH used in the measurements of steady-state activity (pH 7.5), the estimated \(t_{1/2}\) of \(\Delta 27\text{IDO}2\text{Fe}^{3+}-\text{O}_2^{-}\) (48 ± 5 min) was longer than that of \(\text{IDO}1\text{Fe}^{3+}-\text{O}_2^{-}\) \(t_{1/2} 23\) min at pH 7.5, (Hirata et al. 1977); ~7 min at pH 7.0, this study), arguing against enzyme auto-oxidation as a significant determinant of the relatively low L-Trp dioxygenase activity of IDO2.

### 3.3.6 Reactivities of \(\Delta 27\text{IDO}2\) supported by \(\text{H}_2\text{O}_2\)

In addition to its L-Trp dioxygenase activity, \(\Delta 27\text{IDO}2\text{Fe}^{3+}\) catalyzed the one-electron oxidation of ABTS in the presence of \(\text{H}_2\text{O}_2\) (i.e., peroxidation) as indicated by the increase in the absorbance at 735 nm resulting from ABTS\(^{**}\) upon \(\text{H}_2\text{O}_2\) addition. This reaction is also catalyzed by peroxidase enzymes (Childs & Bardsley 1975) and a variety of heme proteins (Radi et al. 1991, Wan et al. 1998) including IDO1 (Lu & Yeh 2011). Steady-state parameters for the formation of ABTS\(^{**}\) by \(\Delta 27\text{IDO}2\) as a function of [\(\text{H}_2\text{O}_2\)] were estimated to be 45 ± 2 s\(^{-1}\) and 35 ± 5 mM for \(k_{\text{cat}}\) and \(K_{\text{m}}\), respectively, at 7.25 mM ABTS (Figure 3.32 A). In comparison to the corresponding parameters reported for IDO1 in the same reaction (Lu & Yeh 2011), \(k_{\text{cat}}\) of \(\Delta 27\text{IDO}2\) is approximately two-fold lower, while the apparent specificity constant with respect to \(\text{H}_2\text{O}_2\) is ~16-fold lower, which suggests that reduction of \(\text{H}_2\text{O}_2\) occurs more efficiently in IDO1Fe\(^{3+}\) than in \(\Delta 27\text{IDO}2\text{Fe}^{3+}\) in the presence of the ABTS substrate.

\(\Delta 27\text{IDO}2\text{Fe}^{3+}\) also catalyzed the oxidation of indole by \(\text{H}_2\text{O}_2\), as indicated by the decrease in the absorbance of indole at 280 nm upon \(\text{H}_2\text{O}_2\) addition, and a corresponding increase in absorbance at 610 nm resulting from its oxidation product indigo blue. HPLC analysis of the reaction mixture further identified monooxygenated and dioxygenated products.
Figure 3.31 Dependence of the rates of $\Delta 27$IDO$2Fe^{3+}$–$O_2^{ullet-}$ auto-oxidation to $\Delta 27$IDO$2Fe^{3+}$ on pH. (A) Representative series of spectra for the conversion of $\Delta 27$IDO$2Fe^{3+}$–$O_2^{ullet-}$ to $\Delta 27$IDO$2Fe^{3+}$ (2.6 µM protein in 10 mM EDTA, 250 nM riboflavin, and 20 mM Bis-tris, pH 6.5, 20 °C) in the presence of catalase (0.5 µg/mL) and SOD (1 unit/mL). The inset depicts changes in absorbance at 521 nm replotted against time (top panel), which were fitted to a mono-exponential function to obtain the auto-oxidation rate constants ($K_{ox}$). Lower panel shows the corresponding residual plot. (B) pH dependence of the half-lives (converted from $K_{ox}$) of $\Delta 27$IDO$2Fe^{3+}$–$O_2^{ullet-}$. The protein is not sufficiently stable beyond pH 6.5–8.5 for data acquisition.
that were also generated in the IDO1-catalyzed reaction. Presumably, these products formed by reactions similar to those responsible for their formation by IDO1 although the characteristics of this reaction by Δ27IDO2 were not defined in the same detail as achieved for IDO1 (i.e., 18O incorporation). As the activity of Δ27IDO2 in catalysis of L-Trp oxidation by O2/O2•− is so low relative to the activity exhibited by IDO1, it was of interest to compare the activities of the two isozymes with respect to their abilities to catalyze indole oxidation by H2O2. The steady-state kinetics of indole oxidation by Δ27IDO2Fe3+ and H2O2 were examined essentially as described for IDO1 (section 3.2.8). The [H2O2]-dependence of the reaction exhibited Michaelis-Menten behavior with \( K_m \) for H2O2 of 1.1 ± 0.1 mM and \( k_{\text{cat}} \) of 2.1 ± 0.1 s\(^{-1}\) at 30 µM indole (Figure 3.32 B), which are comparable to the corresponding values observed for IDO1 (\( K_m \) 0.9 mM H2O2, \( k_{\text{cat}} \) 2.6 s\(^{-1}\) at 25 µM indole). Under the same conditions, horseradish peroxidase (HRP) catalyzed indole oxidation with ≤ 0.5% of the activity (\( k_{\text{cat}} \) 0.01 s\(^{-1}\)) of either IDO isoform. In contrast, IDO1 and Δ27IDO2 exhibited a substantially different [indole]-dependence profiles. Whereas both proteins exhibited biphasic kinetic behavior with increasing concentrations of the substrate, the rates obtained in the Δ27IDO2Fe3+-catalyzed reaction reached a maximum at ~70 µM indole, and decreased at higher [indole] (Figure 3.32 C), while the corresponding rates of IDO1 increased hyperbolically at low [indole] (~0–50 µM) and linearly at higher [indole] (see section 3.2.8). The dependence of activity on [indole] was analyzed by fitting the data to equation 3.3.

\[
v = \frac{V_{\text{max1}}[S]}{K_{m1} + [S] + \frac{K_{m1}[S]^2}{K_{m2}}}
\]

(3.3)

This analysis yielded \( k_{\text{cat1}}, K_{m1}, \) and \( K_{m2} \) values of 1.2 ± 0.1 s\(^{-1}\), 21 ± 4 µM, and 9.6 ± 1 mM, respectively, at constant H2O2 (500 µM). The ratio of \( K_{m2} \) to \( K_{m1} \), which in this case corresponds to the substrate inhibition constant (\( K_s \), see Appendix C for detail), was 460 ± 100 µM. By comparison, fitting only the initial hyperbolic phase to the classic Michaelis-Menten equation
Figure 3.32 Δ27IDO2Fe³⁺-catalyzed oxidation of ABTS and indole by H₂O₂.

(A) Initial rates (v_{ini}, s⁻¹) for the formation of ABTS⁺⁺ as a function of [H₂O₂] determined from the increases in absorbance at 735 nm following rapid mixing of ABTS with Δ27IDO2Fe³⁺ and then H₂O₂ to final concentrations 7.25 mM, 300 nM, and 1–40 mM, respectively. Initial rates (v_{ini}, s⁻¹) of indole oxidation by Δ27IDO2Fe³⁺ (800 nM in 20 mM Tris-HCl, pH 7.5, 25°C) determined from decreases in fluorescence emission at 345 nm (280 nm excitation) as a function of (B) H₂O₂ concentrations at 30 µM indole and (C) indole concentrations at 500 µM H₂O₂. Steady-state kinetic parameters were extracted by fitting the data in (A) and (B) to the Michaelis-Menten equation and data (C) to equation 3.3. This analysis yielded values of [k_{cat} 45 ± 2 sec⁻¹, K_m 35 ± 5 mM], [k_{cat} 2.1 ± 0.1 sec⁻¹, K_m 1.1 ± 0.1 mM], and [k_{cat} 1.2 ± 0.1 sec⁻¹, K_m 21 ± 4 µM, K_{cat} 9.6 ± 1 mM] for (A), (B), and (C), respectively.
yielded $k_{\text{cat}}$ and $K_m$ values of $0.9 \pm 0.1 \, \text{s}^{-1}$ and $13 \pm 2 \, \mu\text{M}$, respectively. In either estimate, the parameters obtained resembled those obtained with IDO1 at similar $[\text{H}_2\text{O}_2]$ ($k_{\text{cat}} \, 1.3 \pm 0.2 \, \text{s}^{-1}$, $K_{m1}$ of $9 \pm 4 \, \mu\text{M}$). Relative to the differences observed in the catalytic rates of L-Trp oxidation between the IDO isozymes, the oxidation of indole by these enzymes proceed at comparable efficiency at low substrate concentrations ($i.e.$, $[\text{indole}] < 30 \, \mu\text{M}$). However, due to the substrate inhibitory kinetic properties of the $\Delta 27$IDO2-catalyzed reaction, significantly higher rates of indole oxidation were obtained with IDO1 at higher concentrations of indole.
Chapter 4: discussion

4.1 β-NADH-peroxidase-oxidase activity of IDO1

The aerobic reaction of IDO1Fe$^{3+}$ with β-NADH affords a convenient means of generating IDO1Fe$^{3+}$−O$_2^•$− in good yield and in a relatively stable form at ambient temperature and atmosphere without the aid of (electron transfer) mediators. Several mechanisms have been described for the oxidation L-Trp by IDO1, and new ideas continue to emerge (Sugimoto et al. 2006, Chung et al. 2008, Lewis-Ballester et al. 2009, Capece et al. 2010). The common starting point in all the mechanisms proposed thus far is the dioxygen-adduct of the enzyme, IDO1Fe$^{3+}$−O$_2^•$−. In practical terms, the enzyme, as isolated (IDO1Fe$^{3+}$), must be activated \textit{in vitro} by reduction and binding of O$_2$ before any of these mechanisms can be evaluated experimentally. Consequently, the IDO1Fe$^{3+}$−O$_2^•$− form of the enzyme is of considerable interest as is the procedure to activate the enzyme experimentally.

In the standard IDO activity assay that was developed originally by Yamamoto and Hayaishi (Yamamoto & Hayaishi 1967), ascorbate and methylene blue were required for L-Trp oxidation. Although IDO1Fe$^{3+}$−O$_2^•$− can be generated with these reagents, spectroscopic analysis of the enzyme in their presence is difficult due to significant overlap of methylene blue absorption band with relevant features of this enzyme intermediate. These authors further examined a range of reducing substrates and mediators that could generate and sustain the otherwise unstable IDO1Fe$^{3+}$−O$_2^•$−. Neither glutathione nor cysteine can substitute for ascorbate although enzyme systems that generate O$_2^•$− can do so \textit{i.e.,} xanthine oxidase and hypoxanthine. While toluidine blue can replace methylene blue, the same is not true for other mediators such as 2,6-dichlorophenolindophenol, potassium ferricyanide, phenazine methosulfate, ferrous sulfate,
or cytochrome c. As a result, the ability of β-NADH to support catalytic turnover of L-Trp by IDO1 provides a new means by which the reactivity of this enzyme can be investigated.

4.1.1 Reactions of β-NADH with heme proteins

In general, electron transfer from β-NADH or β-NADPH to heme proteins involves one or more protein mediators such as a flavoprotein or iron-sulfur protein. These proteins accept two electrons from β-NAD(P)H and are oxidized in two sequential one electron transfers to intermediate acceptors, which then reduce the heme protein through direct electron transfer. The ferredoxin and ferredoxin-NADP⁺ reductase system from spinach, for instance, has been widely used for the reduction of hemoglobin (Hayashi et al. 1973, Nagai et al. 1981). In this case, the flavin adenine dinucleotide (FAD⁺) cofactor of ferredoxin-β-NADP⁺ reductase mediates the two-to-one-electron switch. Other systems such as cytochrome b₅ and β-NADH-cytochrome b₅ reductase or β-NADPH-cytochrome P450 reductase serve similar purpose (Passon & Hultquist 1972, Sannes & Hultquist 1978) and have been used for the reduction of IDO1 and IDO2 in this work and in at least one other study (Austin et al. 2010).

Examples of interactions and reactions of β-NADH with heme proteins with or without the aid of (non-protein) mediators have also been reported. The reduction of ferric myoglobin is catalyzed by β-NADH with the inclusion of EDTA and is accelerated by the addition of flavins, methylene blue or phenazine methosulfate (Brown & Snyder 1969, Kajita et al. 1970), all of which could mediate electron transfer between β-NADH and the protein. These processes occur equally well under illumination or in the dark (Brown & Snyder 1969) and so are distinct from photosensitized reduction processes (vide infra). On the other hand, it has been suggested that the stimulatory effect on the reaction exhibited by EDTA originates from the presence of trace amounts of contaminating iron and formation of the reductant Fe(EDTA)²⁻ (Sannes & Hultquist 1979).
Laser irradiation of β-NADH under anaerobic conditions or in low levels of dioxygen (< 2%) has been shown to reduce cytochrome c (Orii 1993). Czochralska and Lindqvist have reported that photolysis of β-NADH ejects a hydrated electron (e\textsuperscript{−}\textsubscript{aq}) concomitant with the release of a proton to yield β-NAD\textsuperscript{*} (Kosower \textit{et al.} 1978, Czochralska & Lindqvist 1983) as shown in reactions 4.1 and 4.2.

\[
\begin{align*}
\beta\text{-NADH} + h\nu &\rightarrow \beta\text{-NADH}^{**} + e\text{aq}^- \quad (4.1) \\
\beta\text{-NADH}^{**} &\rightarrow \beta\text{-NAD}^* + H^+ \quad (4.2)
\end{align*}
\]

β-NAD\textsuperscript{*} and e\textsuperscript{−}\textsubscript{aq} are strong reductants (\(E_{m} -2.0\) and \(-0.94\) V, respectively) and thus readily reduce a variety of heme proteins or react with O\textsubscript{2} to yield O\textsubscript{2}\textsuperscript{•−} (Yamazaki & Yokota 1967, Willson 1970, Cunningham \textit{et al.} 1985).

An unusual case in which the two electrons from β-NADH are delivered directly to the heme iron (\textit{i.e.}, no mediators or UV irradiation) has been reported for fungal cytochrome P450 nitric oxide reductase (NOR). Cytochrome P450 NOR acquires the two reducing equivalents to convert two molecules of nitric oxide (NO) to nitrous oxide (N\textsubscript{2}O) directly from β-NADH (Nakahara \textit{et al.} 1993, Shiro \textit{et al.} 1995). The mechanism of this reaction presumably involves hydride ion transfer (\textit{i.e.}, two electrons and a proton) to the Fe\textsuperscript{3+}–NO complex to yield an intermediate that is formally (Fe\textsuperscript{3+}–NO)\textsuperscript{2−} (Shiro \textit{et al.} 1995), which then reacts with a second molecule of NO (and H\textsuperscript{+}) to release N\textsubscript{2}O and H\textsubscript{2}O. In this case, the addition of a mediator (\textit{i.e.}, phenazine methosulfate) did not enhance the activity but instead inhibited the reaction (Nakahara \textit{et al.} 1993). This mechanism is supported by the crystal structure cytochrome P450 NOR in complex with NAAD (a NADH analogue), in which the nicotinamide C\textsubscript{4} atom is located near the heme-bound NO presumably facilitating direct hydride ion transfer (Oshima \textit{et al.} 2004).

On the other hand, β-NADH reacts with horseradish peroxidase (HRP) in the presence of O\textsubscript{2} to form the HRPFe\textsuperscript{2+–O\textsubscript{2}} (or HRPFe\textsuperscript{3+–O\textsubscript{2}\textsuperscript{•−}}) derivative (also referred to as HRP-Compound
III) as first observed by Yokota and Yamazaki (Yamazaki & Yokota 1965, Yokota & Yamazaki 1965). Nominally, the net reaction leads to reduction of O$_2$ to H$_2$O (reaction 4.3), hence this reaction is known in the literature as the peroxidase-oxidase reaction.

$$2 \beta\text{-NADH} + O_2 \rightarrow 2 \beta\text{-NAD}^+ + 2 H_2O$$ (4.3)

Under a continuous supply of O$_2$, this reaction exhibits unusual oscillatory kinetics that stem from accumulation, decay, and re-accumulation of HRP-Compound III (Yamazaki & Yokota 1965, Yamazaki & Yokota 1967). These events in turn coincide with fluctuations in the concentration of O$_2$ in solution. Other peroxidases, including lactoperoxidase and myeloperoxidase, have also been shown to exhibit similar oscillatory kinetics (Nakamura et al. 1969, Valeur & Olsen 1996, Brasen et al. 2004). Extensive modeling efforts have yielded rate constants for many of the numerous reactions involved in this complex process (Scheeline et al. 1997, Kirkor et al. 2000). The principal reactions are discussed below (section 4.1.2) insofar as they are pertinent to the results observed with IDO1 (section 4.1.3).

### 4.1.2 Mechanism of β-NADH oxidation by HRP

The generally accepted mechanism for the β-NADH peroxidase-oxidase activity of HRP (Halliwell & De Rycker 1978, Scheeline et al. 1997) is initiated by the slow auto-oxidation of β-NADH by dissolved O$_2$ ($3 \times 10^{-6}$ s$^{-1}$ at pH 5.6, 150 µM O$_2$, (Yokota & Yamazaki 1977)), which leads to accumulation of catalytic amount of H$_2$O$_2$ (reaction 4.4).

$$\beta\text{-NADH} + O_2 + H^+ \rightarrow \beta\text{-NAD}^+ + H_2O_2$$ (4.4)

The H$_2$O$_2$ thus generated is reduced in the classic peroxidase reaction cycle (Dunford 2010), in which HRPFe$^{3+}$ reacts with H$_2$O$_2$ to yield a Fe$^{4+}$=O porphyrin cation radical enzyme intermediate (i.e., HRP-Compound I) and H$_2$O (see section 4.2.3 for more details regarding this step). HRP-Compound I then abstracts one electron from β-NADH to yield HRPFe$^{4+}$=O (i.e., HRP-Compound II) and β-NADH$^{•+}$, which rapidly deprotonates to the free radical. A second
\( \beta \)-NADH reduces HRP-Compound II to the ferric enzyme to yield again \( \beta \)-NADH\(^+\)/NAD\(^-\), and in the process releasing the ferryl oxygen as \( \text{H}_2\text{O} \) (two \( \text{H}^+ \) are supplied by \( \beta \)-NADH). Once formed, \( \beta \)-NAD\(^-\) can reduce: (a) HRP-Compound II to HRPFe\(^{3+}\) (and \( \text{OH}^- \)), (b) HRPFe\(^{3+}\) to HRPFe\(^{2+}\), and (c) dissolved \( \text{O}_2 \) to \( \text{O}_2^- \), as shown in reactions 4.5–7.

\[
\begin{align*}
\beta \text{-NAD}^- + \text{HRP-Compound II} & \rightarrow \text{NAD}^+ + \text{HRPFe}^{3+} + \text{OH}^- & (4.5) \\
\beta \text{-NAD}^- + \text{HRPFe}^{3+} & \rightarrow \text{NAD}^+ + \text{HRPFe}^{2+} & (4.6) \\
\beta \text{-NAD}^- + \text{O}_2 & \rightarrow \text{NAD}^+ + \text{O}_2^- & (4.7)
\end{align*}
\]

The Fe\(^{2+}\) and Fe\(^{3+}\) enzyme generated in the above reactions then bind, respectively, \( \text{O}_2 \) and \( \text{O}_2^- \) to produce the oxygenated enzyme, HRP-Compound III (reactions 4.8 and 4.9).

\[
\begin{align*}
\text{HRPFe}^{2+} + \text{O}_2 & \Leftrightarrow \text{HRP-Compound III} & (4.8) \\
\text{HRPFe}^{3+} + \text{O}_2^- & \Leftrightarrow \text{HRP-Compound III} & (4.9)
\end{align*}
\]

HRP-Compound III can auto-oxidize to the ferric state to re-enter the peroxidase reaction cycle (reverse of reaction 4.9), releasing \( \text{O}_2^- \) in the process. In addition to reaction 4.4, a second pathway leading to the generation of \( \text{H}_2\text{O}_2 \) is made available by \( \text{O}_2^- \). At acidic pH, the disproportionation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) occurs by the protonated species perhydroxyl radical \( \text{HO}_2^- \) (reaction 4.10, (Bielski & Allen 1977), \( pK_a \) 4.88 (Land & Swallow 1971)), or by reaction of the protonated and de-protonated forms with each other (reaction 4.11, (Bielski & Schwarz 1968)).

\[
\begin{align*}
\text{HO}_2^- + \text{HO}_2^- & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 & (4.10) \\
\text{HO}_2^- + \text{O}_2^- + \text{H}_2\text{O} & \rightarrow \text{H}_2\text{O}_2 + \text{OH}^- & (4.11)
\end{align*}
\]

Reactions (4.4, 4.10, and 4.11) account for the total production of \( \text{H}_2\text{O}_2 \) and are sufficient to allow for continuous oxidation of \( \beta \)-NADH and accumulation of HRP-Compound III as a by-product, even without external sources of \( \text{H}_2\text{O}_2 \). The peroxidase-oxidase reaction is essentially a radical chain reaction; a number of additional reactions (i.e., chain termination) are required to model the oscillatory nature of the reaction fully. Consideration of these reactions is not included.
in the current discussion, but they are reviewed thoroughly by Scheeline and authors (Scheeline et al. 1997).

### 4.1.3 Mechanism of β-NADH oxidation by IDO1

β-NADH exhibits little or no reactivity with IDO1 under anaerobic conditions unless a mediator (*i.e.*, phenazine methosulfate or methylene blue) is present, implying that O₂ acts as more than a ligand in the generation of IDO1Fe³⁺–O₂⁻. While IDO1 continuously oxidizes β-NADH under aerobic conditions, this process is strongly inhibited by catalase and SOD, suggesting that O₂⁻ and H₂O₂ are required for initiation and/or catalysis of β-NADH oxidation by the enzyme. As described earlier in the discussion of the HRP-oxidase system, O₂⁻ can be obtained by reaction of β-NAD⁺ with O₂ (reaction 4.7). As the reaction of β-NADH with IDO1 proceeds in the dark, photolytic production of NAD⁺ is negligible in this reaction. Most likely then, β-NAD⁺ is generated by reaction of β-NADH with ferryl derivatives of IDO1, in a manner analogous to peroxidases (reactions 4.12–14).

\[
\begin{align*}
\text{IDO1Fe}^{3+} + \text{H}_2\text{O}_2 & \rightarrow \text{IDO1-Compound I} + \text{H}_2\text{O} \\
\text{IDO1-Compound I} + \beta-\text{NADH} & \rightarrow \text{IDO1-Compound II} + \beta-\text{NAD}^+ + \text{H}^+ \\
\text{IDO1-Compound II} + \beta-\text{NADH} & \rightarrow \text{IDO1Fe}^{3+} + \beta-\text{NAD}^+ + \text{OH}^- 
\end{align*}
\]

That H₂O₂ required for the above reactions is generated *in situ* (reactions 4.4, 4.10, 4.11) is consistent with the observation that aged aerobic solution of β-NADH, which accumulate increasing amounts of H₂O₂ over time, accelerate reaction of β-NADH with IDO1. Moreover, the lower yield of IDO1Fe³⁺–O₂⁻ at acidic pH is also consistent with the faster rate of disproportionation of HO₂⁺ to H₂O₂, which competes with the binding of O₂⁻ to IDO1Fe³⁺. Even crystalline β-NADH powder is not stable to oxidation by O₂ owing to the hygroscopic nature of

---

¹ NADH⁺ deprotonates readily under typical reaction conditions (pKₐ ≈ 4, Martens and Verhoeven 1981). Proton transfer to IDO1 is less likely in view of its lack of suitable active-site base.
this reagent. As noted previously, a peroxide adduct of β-NADH forms slowly in aerobic solutions of β-NADH, and this adduct appears to form to varying extent even in the solid state (Bernofsky & Wanda 1982). Consequently, different samples of β-NADH can exhibit significant variation in their reactivity with IDO1 depending on their storage conditions.

Evidence that IDO1 possesses peroxidase activity has been reported for the substrates guaiacol and ABTS (Shimizu et al. 1978, Lu & Yeh 2011). Because the midpoint potential of the one-electron oxidation couple of ABTS is +472 mV versus SHE (Bourbonnais et al. 1998), it is reasonable to assume that the midpoint potentials of IDO1-Compound I or II are greater and very likely similar to those of HRP (i.e., around +0.9 V, (Hayashi & Yamazaki 1979)). The midpoint potential of the β-NADH/NAD$^{\bullet\bullet}$ couple has been reported to be +282 mV versus SHE (Wardman 1989). From a thermodynamic standpoint then, one-electron reduction of IDO1-Compound I or II by β-NADH is favourable energetically. Notably, although two sequential one-electron-reactions are proposed for this process, analogy with the minimal model for β-NADH-peroxidase-oxidase activity requires only the formation of IDO-Compound II. As noted in Chapter 1, a ferryl intermediate (i.e., IDO1Fe$^{4+}$=O) has been detected by resonance Raman spectroscopy upon reaction of IDO1Fe$^{3+}$ with H$_2$O$_2$ (Lu & Yeh 2011). Conceptually, IDO1-Compound I may form as a precursor to IDO1-Compound II, but this intermediate has eluded spectroscopic detection so far although there is some evidence supporting this idea as discussed later (see section 4.2).

Despite these reactivities, IDO1 is susceptible to H$_2$O$_2$-induced damage in the absence of reducing substrates. H$_2$O$_2$ at relatively low concentrations reportedly oxidizes as many as five of the eight Cys residues of IDO1 to sulfinic or sulfonic acid while at greater concentrations peroxide resulted in the destruction of the heme group (Poljak et al. 2006). Although the formation of IDO1Fe$^{3+}$–O$_2$$^{\bullet\bullet}$ by β-NADH involves H$_2$O$_2$, loss of IDO1 activity does not occur
under typical reaction conditions used here (i.e., pH 7 to 7.5, up to 500 µM β-NADH), presumably because the rate at which H₂O₂ is generated in solution is less than or equal to the rate at which it is consumed in the peroxidase cycle. Limiting conditions, however, include reactions carried out at acidic pH, high [β-NADH] (> 1 mM), or with NMNH instead of β-NADH, because under these conditions an increase of protein aggregation is evident from the increasing light-scattering of the solution.

The principal reactions of the β-NADH-peroxidase-oxidase system are summarized and adapted for IDO1 in Figure 4.1. In this reaction scheme, the direct reduction of the IDO1Fe³⁺ by β-NAD⁺ and subsequent binding of O₂ by IDO1Fe²⁺ probably represents only a minor pathway to IDO1Fe³⁺–O₂⁻. Although it is conceivable that β-NAD⁺ reduces IDO1Fe³⁺, O₂ in solution competes with the protein for reaction with β-NAD⁺, hence little IDO1Fe²⁺ accumulates following addition of β-NADH to IDO1. Likewise, the reaction of O₂ with IDO1Fe²⁺ to form IDO1Fe³⁺–O₂⁻ is probably also a minor component of the IDO1-β-NADH system. The corresponding reaction in the HRP-β-NADH system presumably contributes to the the oscillatory kinetics of HRP-Compound III accumulation (Scheeline et al. 1997). Oscillatory kinetic has not been observed with IDO1 in this work, but the range of reaction conditions explored so far is insufficient to rule out such behaviour.

Finally, it is notable that even though this discussion has concerned IDO1 only, the related enzyme IDO2 also catalyzes the aerobic oxidation of β-NADH with concomitant formation of the oxygenated enzyme, presumably by the mechanism described here for IDO1.

4.1.4 Interactions of β-NADH and IDO1

Although molecular-docking algorithms predict that binding of the nicotinic acid ring and adenine ring of β-NADH in the distal pocket of IDO1 are equally favourable, no appreciable
Figure 4.1 Reactions involved in the β-NADH-peroxidase-oxidase activity of IDO1. Cmpd1 and Cmpd2 denote IDO1-Compound I and IDO1-Compound II, respectively.
changes were detected in the electronic absorption spectrum of IDO1Fe$_{3+}$ or in the fluorescence emission spectrum of β-NADH upon mixing of these two components. Consequently, no simple means of evaluating the affinity of the distal pocket (or any other site on the protein) for β-NADH is available. However, conversion of IDO1Fe$_{3+}$ to IDO1Fe$_{3+}$−O$_2^•$ by a peroxidatic mechanism of the type described above does not require high affinity binding or that the nicotinic acid ring bind in the distal heme pocket. Instead, rapid electron transfer from β-NADH to the ferryl heme of IDO1 and then from β-NAD$^•$ to O$_2$ is the minimal requirement. The rate of electron transfer, according to the Marcus theory (Marcus 1956), is in part dependent on the thermodynamic driving force ($\Delta G$) of the reaction, which is defined by the difference of the midpoint potentials of the electron donor and acceptor (as noted earlier in section 4.1.3), and the distance separating the donor and acceptor. The closest approach of the nicotinamide ring of β-NADH to the heme iron (~4.5 Å) defined by the simulations described above is too great to establish direct electronic interaction with the heme iron although it is possible that reduction of the donor-acceptor distance by the bridging ferryl oxygen could reduce this separation thereby facilitating electron transfer. However, it is also likely that the significant solvent-accessibility of the heme group of IDO1 enables formation of a great number of effective alternative donor-acceptor geometries and related electronic coupling pathways (e.g., contact through the exposed $\gamma$-meso carbon edge or 7-propionate group of the heme). As a result, transient complexes conducive to electron transfer may result from ligand diffusion at the surface of IDO1. This process could be assisted by Arg248 near the entry to the heme cavity, which may participate in salt bridge or cation-π interactions with the pyrophosphate or pyridine moiety of β-NADH, respectively.

As described in Chapter 2, the method developed for purification of IDO1 (and IDO2) during this work includes a chromatographic step involving Cibacron blue 3G-A agarose resin.
Cibacron blue 3G-A resembles closely NAD(P)H insofar as it has been shown to exhibit a strong affinity for known NAD(P)H-binding proteins. As a result, the immobilized dye has found popular application as a chromatographic affinity agent for the identification and purification of proteins possessing the dinucleotide fold (Thompson & Stellwagen 1976, Stellwagen 1990). The observation that IDO1 binds to Cibacron blue 3G-A agarose resin and that it can be eluted with a gradient of salt, NAD\textsuperscript{+}, or l-Trp suggests strongly that β-NADH exhibits an affinity for the active site of IDO1 despite the absence of a dinucleotide fold at this site.

Reduced nicotinamide mononucleotide (NMNH) is more efficient in promoting IDO1Fe\textsuperscript{3+}–O\textsuperscript{2−} formation than is the dinucleotide. In solution, β-NADH exists as an equilibrium mixture of extended and folded conformations (McDonald et al. 1972, Hull et al. 2001). In contrast, enzyme-bound β-NADH occurs in the extended conformation, as exemplified in the three-dimensional structures of the cytochrome P450 NOR-NAAD complex (Oshima et al. 2004) and dehydrogenases with bound β-NAD\textsuperscript{+} (Grau et al. 1981). O\textsuperscript{2−} and HO\textsuperscript{2−} radicals reportedly oxidize enzyme-bound β-NADH rapidly, whereas free β-NADH is impervious to attack by these radicals (Bielski & Chan 1980). Presumably, this enhanced reactivity towards reactive oxygen species results from the extended conformation when β-NADH is bound to protein. This expectation is consistent with the increased rate of IDO1Fe\textsuperscript{3+}–O\textsuperscript{2−} formation observed following hydrolysis of β-NADH with phosphodiesterase because the NMNH released in this manner lacks the adenine-pyridine ring stacking interaction of the folded conformation. As a result, reaction of NMNH with dissolved O\textsubscript{2} is relatively more facile or, alternatively, the smaller molecule is better able to enter the heme cavity of IDO1.

4.1.5 Biological implications of the β-NADH-peroxidase-oxidase activity of IDO1

Ultimately, the activation of IDO1 by β-NADH raises an issue regarding the potential physiological implications of IDO1 β-NADH peroxidase-oxidase activity in L-Trp metabolism.
In considering this question it is useful to consider similar reactions catalyzed by the related enzyme, TDO. It is well known that TDO can be activated *in vitro* to the ferrous state by H$_2$O$_2$ and L-Trp (Fu *et al.* 2011). The mechanism of this activation was demonstrated to proceed by a cytochrome c peroxidase Compound I-like derivative of TDO with a ferryl heme and a protein-centered radical that is generated by reaction of the enzyme with H$_2$O$_2$. This derivative is then reduced by L-Trp in one-electron and then two-electron steps to yield the (activated) ferrous protein and L-Trp oxidation by-products (Fu *et al.* 2011). These authors suggested that an elevated concentration of H$_2$O$_2$ resulting from oxidative stress could lead to highly-oxidized forms of TDO in hepatocytes and that these intermediates could be converted subsequently to the ferrous state by reaction with L-Trp via the above pathway. In contrast, IDO1 oxidized by H$_2$O$_2$ does not readily react with L-Trp to form the ferrous enzyme in the same manner. Nonetheless, because elevated IDO1 activity is associated with a diminished immune response, it seems reasonable to propose that IDO1 may also function in an oxidizing environment. One means by which IDO1 activity could be maintained under these conditions could be afforded by reaction with $\beta$-NADH, which, as demonstrated *in vitro*, results in the formation of the active IDO1$\text{Fe}^{3+}$O$_2$$\cdot^-$ intermediate to oxidize L-Trp to $N$-FK provided that at least catalytic amounts of H$_2$O$_2$ are present.

Nearly 50 years ago, Nishizuka and Hayaishi reported evidence that L-Trp metabolism leads to formation of niacin ribonucleotide, a key intermediate in the synthesis of NAD$^+$ (Nishizuka & Hayaishi 1963). This proposal explained the observation in several species, including humans, that an adequate amount of dietary L-Trp eliminates the dietary requirement for the vitamin niacin. The $\beta$-NADH peroxidase-oxidase activity of IDO1 raises the intriguing possibility of another metabolic relationship between NAD$^+$ and L-Trp metabolism in which reduced pyridine dinucleotide activates the first and rate-limiting reaction in the metabolic pathway that leads to its own biosynthesis.
4.2 Indole-peroxygenase activity of IDO1

4.2.1 Reactivity of IDO1 with H$_2$O$_2$

Early work by Hayaishi and colleagues reported in passing that IDO1 possesses peroxidase activity (Shimizu et al. 1978). Subsequently, IDO1 was shown to catalyze the H$_2$O$_2$-dependent $N$-demethylation of benzphetamine and hydroxylation of aniline (Takikawa et al. 1983) although it does not catalyze oxidation of L-Trp by H$_2$O$_2$ at appreciable rates (Hayaishi 1976, Ferry et al. 2005, Lu & Yeh 2011). More recently, the peroxidase activity of IDO has attracted renewed attention that has led more recently to the current work concerning the $\beta$-NADH peroxidase-oxidase activity. The work of Takikawa and co-workers concerning the oxidation of benzphetamine and aniline provided the first insights concerning the role of oxygen sources other than O$_2^•-$ in substrate oxidation by IDO1 (Takikawa et al. 1983). These authors showed that benzphetamine reacts anaerobically with IDO1 and H$_2$O$_2$ in approximately one-to-one correspondence to yield norbenzphetamine and formaldehyde. Cytochrome P450 monooxygenases had been shown previously to catalyze the same reaction with the transfer of one oxygen atom from the oxidant (O$_2$ or H$_2$O$_2$), so it was proposed that IDO1 catalyzes the reaction in a similar manner (Takikawa et al. 1983). Although this conclusion is reasonable, it is worth noting that $N$-dealkylation of tertiary amines is also commonly catalyzed by peroxidases such as HRP (Kedderis & Hollenberg 1985, Kedderis et al. 1986, Hollenberg 1992), even though benzphetamine, specifically, is not a substrate for HRP peroxidation (Nordblom et al. 1976). Identification of the source of the oxygen transferred in the cytochrome P450- and peroxidase-catalyzed reactions through use of $^{18}$O-labelled peroxide is complicated by the rapid oxygen exchange between the formaldehyde product and H$_2$O. This problem was circumvented by Kedderis and Shea, who used $N$-methylcarbazole as substrate to demonstrate that only a fraction (~20%) of the oxygen in the product is derived from the oxidant in HRP-catalyzed reactions.
(Kedderis et al. 1986). In contrast, nearly all of the oxygen (>95%) in the product of the cytochrome P450-catalyzed reactions is derived from the oxidant (Shea et al. 1982).

In the same report, Takikawa also described the peroxide-dependent hydroxylation of aniline by IDO1 (Takikawa et al. 1983). Curiously, unlike benzphetamine, hydroxylation of aniline required aerobic conditions and $O_2^{-}$•. While the source of the oxygen incorporated into the product of this peroxide-dependent reaction of IDO1 remains uncertain, the limited literature that is available implies that IDO1 may be capable of processing alternative substrates in the presence of $H_2O_2$. Presumably, the relatively high accessibility of the IDO1 active-site (Sugimoto et al. 2006) contributes significantly to versatility in IDO1 reactivity.

4.2.2 Oxidation of indole by heme proteins

Aside from L-Trp, the dioxygenase activity of IDO1 extends to oxidation of other indoleamines such as 5-hydroxy-L-Trp, serotonin, and tryptamine while indole, 3-methylindole, and indoleacetic acid are not substrates (Hayaishi 1976, Hirata et al. 1977, Shimizu et al. 1978). Although indole can apparently bind to $IDO1Fe^{3+}–O_2^{-}$• and the $IDO1Fe^{2+}–CO$ complex (Sono 1989), binding of indole to $IDO1Fe^{3+}–O_2^{-}$• does not influence the rate of autoxidation of this intermediate to $IDO1Fe^{3+}$ nor does it result in the oxidation of indole (Hirata et al. 1977). Although oxidation of indole by IDO enzymes has not been reported previously, several heme enzymes are known to do so. Specifically, HRP (Holmes-Siedle & Saunders 1957), chloroperoxidases (Corbett & Chipko 1979), a number of cytochromes P450 (Gillam et al. 2000) and their variants (Nakamura et al. 2001, Li et al. 2008) have all been reported to exhibit this activity. Also, several non-heme-containing oxygenases (Ensley et al. 1983, McClay et al. 2005)) catalyze this reaction. Some of these are discussed below.

HRP catalyzes the oxidation of indoles and a number of indoleamines by $H_2O_2$ (i.e., melatonin and L-Trp, (Ximenes et al. 2001, Ximenes et al. 2001)). Among the indolic substrates
of HRP, oxidation of the major plant growth hormone indole-3-acetic acid (IAA) is the best documented (Kenten 1955, Fox et al. 1965, Hinman & Lang 1965, Ricard & Nari 1967, Yamazaki & Yamazaki 1973, Kobayashi et al. 1984) and is yet another example of the peroxidase-oxidase activity of HRP insofar as H$_2$O$_2$ is not required to initiate the reaction but is instead generated from the reactions of IAA radicals with O$_2$. Aside from IAA, oxidation of indole and indoleamines by HRP follows the orthodox peroxidase reaction cycle, wherein the initial step proceeds by electron abstraction from indole to yield the indolyl cation radical. The indolyl radicals can: (a) react with other radicals to result, ultimately, in the trimeric product 2,2’-bis-(3-indolyl-)indoxyl (Nakatani & Dunford 1980), or (b) react with O$_2$ or O$_2$•$^-$ to form the indole-peroxyl radical or indole-hydroperoxide, respectively (Winterbourn & Kettle 2003). The indole-peroxo or hydroperoxo intermediate is thought to re-arrange through a 3α-hydroperoxyhexahydropyrroloindole intermediate to yield the opened pyrrole ring products that are structurally analogous to N-FK (Nakagawa et al. 1977).

A number of cytochromes P450 (i.e., CYP2E1, CYP2A6, and engineered mutants of CYP101, CYP102A1) are known to catalyze oxidation of indole. The mixture of products produced in these reactions are distinct from those produced by HRP, but similar to those produced by IDO1 (Li et al. 2000, Gillam & Guengerich 2001, Nakamura et al. 2001, Li et al. 2008). These reactions initially generated interest due to their production of the industrially useful dyes indigo blue and indirubin; the latter is also an active constituent in traditional Chinese medicine used in the treatment of chronic leukemia (Hoessel et al. 1999). Other products that have been reported include 2- and 3-oxoindole, 5- and 6-hydroxyindole, 2-hydroxy-3-oxoindole, indole-2,3-dione, and oxazolobisindole (Gillam et al. 2000).

The observation of various mixtures of indole oxidation products in reactions of cytochromes P450 and peroxidases is an example of the often divergent catalytic fates of identical substrates oxidized by these two classes of enzyme (i.e., cytochromes P450 transfer
oxygen to substrate, while peroxidases generally abstract electrons from substrate). Nonetheless, these two classes of heme enzyme are similar in that both involve oxidation of the resting ferric enzyme to Compound I, the intermediate generally regarded as the principal reactive species (Slijgar et al. 2005, Makris et al. 2006, Dunford 2010, Rittle & Green 2010).

4.2.3 Mechanisms of indole oxidation by IDO1

In the current study, IDO1 was found to oxidize IAA under aerobic conditions presumably via a reaction chain similar to that which lead to the reduction and oxygenation of the protein upon exposure to aerobic solutions of β-NADH. The products of this reaction are the same as those obtained with HRP (i.e., indole-3-methanol and indole-3-aldehyde), but this reaction was not studied in detail although discovery of this reaction led to the subsequent studies concerning the oxidation of indole by IDO1. In other, related initial studies, IDO1 was found to catalyze slow oxidation of L-Trp by H₂O₂ over several hours, a reaction time much greater than normally used to monitor IDO1 dioxygenase activity. Approximately 20% of substrate was generally converted to N-FK after overnight incubation periods. This reaction likely occurred by the peroxidative mechanism described above for HRP.

Although IDO1 is unable to oxidize indole by O₂ as previously noted, the enzyme is able to catalyze the oxidation of this substrate in the presence of H₂O₂. This reaction generated monooxygenated products 2-oxoindole, 3-oxoindole, and a mixture of dioxygenated products with the more highly oxidized products becoming more prominent as the ratio of [H₂O₂] to [indole] was increased. This reaction proceeded under anaerobic conditions and was not inhibited by SOD, D-mannitol or lidocaine, indicating that IDO1-catalyzed indole oxidation does not require dissolved O₂ and that it does not involve O₂•⁻, OH• or singlet oxygen outside the heme cavity. The consumption of indole and H₂O₂ proceeded at nearly stoichiometric proportions at moderate concentrations of H₂O₂. The oxygen incorporated into the monooxygenated products
was shown by $H_2^{18}O_2$ labeling studies to originate from this oxidant. The catalytic cycle proposed for this reaction starts and ends with the ferric enzyme, thereby accounting for two oxidizing equivalents from $H_2O_2$. However, 3-oxoindole, a product of the main reaction, can reduce IDO$Fe^{3+}$ to the ferrous state depending on solution conditions (i.e., dissolved $[O_2]$ and $[H_2O_2]$). The characteristics of this reaction thus resemble closely the activation of cytochromes P450 by the $H_2O_2$-shunt pathway (Hrycay et al. 1975, Nordblom et al. 1976). Based on these similarities, IDO1 activation appears to proceed via an analogous mechanism that leads to a reactive enzyme intermediate that transfers oxygen from the peroxide to indole (i.e., peroxygenation). As discussed briefly in Chapter 1, the peroxide shunt pathway bypasses the requirement to supply the enzyme $2e^-$, $2H^+$, and $O_2$ during the normal catalytic cycle of cytochromes P450 by providing all these ingredients in the form of a single $H_2O_2$ or organic hydroperoxides.

The mechanism by which higher oxidation states of IDO1 heme are formed following reaction with hydroperoxides remains to be elucidated. Based on structural and mutagenic studies of cytochrome c peroxidase and HRP, it is hypothesized that as the hydroperoxide approaches the heme iron, a distal base abstracts a proton from peroxide, and the resulting hydroperoxy anion binds (weakly) to the heme iron to form a ferric-hydroperoxo intermediate ($Fe^{3+}$–OOH or $Fe^{3+}$–OOR) (Poulos & Kraut 1980, Dunford 2010). More recent QM/MM studies suggest that a water molecule could be involved in proton transfer as well (Derat et al. 2007). Subsequent cleavage of the O–O bond of $Fe^{3+}$–OOH has been rationalized with the push-pull concept (Finzel et al. 1984, Dawson 1988, Poulos 1988) wherein the proximal histidine ligand donates electron density through the iron atom to the $\pi$-antibonding orbitals of the O–O bond (i.e., the push effect). At the same time, a distal polar residue protonates the distal peroxide oxygen to facilitate heterolysis of this bond and to release $H_2O$ or ROH as shown in reaction 4.16 (i.e., the pull effect). On the other hand, if electron-donating substituents are attached to the distal
peroxide oxygen (i.e., the pull effect now becomes a push effect), homolytic cleavage of the O–O bond is favoured instead resulting in the release of HO\(^\bullet\) or RO\(^\bullet\) as depicted in reaction 4.17.

\[
\begin{align*}
\text{Fe}^{3+} &- \text{OOR} + \text{H}^+ \rightarrow \text{Compound I} + \text{ROH} \quad (4.16) \\
\text{Fe}^{3+} &- \text{OOR} \rightarrow \text{Compound II} + \text{RO}^\bullet \quad (4.17)
\end{align*}
\]

4.2.3.a Oxidation of indole by IDO1-Compound II

As previously noted, spectroscopic evidence for only IDO1-Compound II (i.e., IDO1Fe\(^{4+}=\text{O}\)) has been obtained by resonance Raman upon reaction of IDO1Fe\(^{3+}\) with H\(_2\)O\(_2\) (Lu & Yeh 2011), so the reactions of IDO1 with indole in the context of a reactive IDO1-Compound II intermediate resulting from homolytic O–O bond cleavage (reaction 4.17) are considered first. At least three reaction pathways involving Compound II are possible (reactions 4.18–21, indole is represented as Ind(H) and 2- and 3-oxoindole as Ind–OH):

\[
\begin{align*}
\text{IDO1-Compound II} + \text{Ind(H)} + \text{H}^+ &\rightarrow \text{IDO1Fe}^{3+} + \text{Ind(H)}^\bullet + \text{OH}^- \quad (4.18) \\
\text{IDO1-Compound II} + \text{Ind(H)} &\rightarrow \text{IDO1Fe}^{2+} + \text{Ind–OH} \quad (4.19) \\
\text{IDO1-Compound II} + \text{Ind(H)}^{\bullet \bullet} &\rightarrow \text{IDO1Fe}^{3+} + \text{Ind–OH} \quad (4.20) \\
\text{IDO1-Compound II} + \text{Ind}^\bullet + \text{H}^+ &\rightarrow \text{IDO1Fe}^{3+} + \text{Ind–OH} \quad (4.21)
\end{align*}
\]

Reaction 4.18 is a typical peroxidatic reaction resulting in the formation of indolyl cation radicals as described earlier for HRP. Formation of 2- or 3-oxoindole from reactions of indolyl radicals with O\(_2\) is, however, largely excluded on the basis of near stoichiometric incorporation of oxygen from H\(_2\)O\(_2\) in these products. Moreover, in view of the midpoint potential of indole \((E_m\) of indole 0.97 V at pH 7, (Harriman 1987)), one-electron oxidation processes are probably kinetically insignificant by analogy to L-Trp which has a similar \(E_m\) values (1.015 V, (Harriman 1987)) and yet is minimally reactive. Alternatively, the reaction can proceed by direct electrophilic addition of the IDO1-Compound II oxygen to electron-rich indole according to reaction 4.19, which terminates with IDO1 in the ferrous state. Under aerobic conditions, binding
of O$_2$ at the heme iron to form the relatively stable IDO1Fe$^{3+}$–O$_2$$^•$– would be expected to compete with binding of H$_2$O$_2$ and thus would interfere with further reactions with indole. However, no significant differences in indole oxidation kinetics were found between reactions carried out under air saturated and anaerobic conditions. Moreover, the enzyme retained predominantly ferric character during aerobic and anaerobic steady state turnover, indicating that this reaction pathway does not contribute significantly to the oxidation of indole. It is possible that oxygen transfer occur from IDO1-Compound II to indolyl cation radicals or free radicals instead (reactions 4.20 and 4.21, respectively), which would proceed with regeneration of the ferric enzyme. In this case, the substrate radical originate from a second equivalent of IDO1-Compound II, which is unlikely due to the near 1:1 stoichiometry of indole to H$_2$O$_2$; reaction would produce maximally 50% of possible products. Similarly, formation of indole free radicals by reaction with HO$^•$ radicals released during the initial peroxide bond scission (reaction 4.17) is considered unlikely owing to the lack of inhibition by HO$^•$ scavengers although such a mechanism could operate if the reaction occurs solely within the active site of the enzyme.

4.2.3.b Oxidation of indole by IDO1-Compound I

The reaction of IDO1Fe$^{3+}$ with cumene hydroperoxide yielded cumene alcohol and acetophenone in approximately 3:1 ratio as a result of the involvement of oxy anions (via reaction 4.16) and oxy radicals (via reaction 4.17), respectively. This observation indicates that heterolytic O–O bond cleavage of cumene hydroperoxide occurred more readily than homolytic O–O cleavage with IDO1. One may anticipate that reactions of IDO1Fe$^{3+}$ with H$_2$O$_2$ also dissociates proceed predominantly by a heterolytic mechanism, because the electron donating cumyl substituent is likely more susceptible to homolytic O–O bond cleavage than is H$_2$O$_2$ as rationalized by the *push-pull* model. The stoichiometric transfer of oxygen to indole by IDO1, which initiated and terminated with the enzyme in the ferric state, is consistent with the
involvement of a reactive enzyme intermediate with an iron atom that is formally two equivalents more oxidized than the ferric state (reaction 4.22), which is only possible by the heterolytic scission of peroxide.

\[
\text{IDO1-Compound I} + \text{Ind(H)} \rightarrow \text{IDO1Fe}^{3+} + \text{Ind-OH} \quad (22)
\]

A heterolytic O–O bond cleavage mechanism is not immediately obvious in view of the heme environment of IDO1. In contrast to HRP or cytochrome \(c\) peroxidase, IDO1 lacks an effective polar residue on the distal side of the heme that is, in addition, within suitable hydrogen-bonding distance to serve as a general acid-base catalyst. Hydrogen bonding interaction between \(\text{H}_2\text{O}_2\) and indole bound at the distal pocket could stabilize charge separation during heterolytic cleavage, but direct protonation via the indole nitrogen proton is unlikely because of its high \(pK_a\) (\(pK_a 17, (\text{Yagil 1967})\)). The manner in which water binds in the distal heme pocket of IDO1Fe\(^{3+}\) is not known. Nevertheless, Davydov and co-workers have shown by \(^1\text{H-ENDOR}\) spectroscopy that the proton in the IDO1Fe\(^{3+}\)-OOH exchanges with solvent (Davydov et al. 2010), so it is conceivable that water bound in the active site provides the hydrogen bonding interactions required to facilitate heterolytic O–O bond cleavage. As noted in Chapter 1, resonance Raman data suggest that the proximal His–Fe bond exhibits peroxidase-like character due to the partially anionic proximal histidine (Terentis et al. 2002). This similarity to peroxidase enzymes suggests that the heme environment of IDO1 exhibits some characteristics that are conducive to a heterolytic O–O bond cleavage mechanism.

As noted earlier, a Compound I derivative of IDO1 has not been detected directly although such a derivative with a protein-centered radical analogous to Compound I of cytochrome \(c\) peroxidase has been identified for the related enzyme, TDO (Fu et al. 2011). Attempts to observe IDO1-Compound I have been reported in resonance Raman and EPR studies by Lu, Davydov, and co-workers (Davydov et al. 2010, Lu & Yeh 2011). In the latter work, the
IDO1Fe$^{3+}$−OOH intermediate was prepared by radiolytic reduction of IDO1Fe$^{3+}$−O$_2^*$ at 77 K. Potentially, annealing of this species could have permitted detection of IDO1-Compound I, but upon injection of a second electron by γ-radiation, the low-spin ferric form (OH$^-$ bound) was obtained (Davydov et al. 2010). The authors interpreted these observations as resulting from conversion of IDO1Fe$^{3+}$−OOH to the EPR-silent IDO1-Compound II during the annealing stage, followed by reduction and protonation to yield the ferric enzyme. Interestingly, analogous experiments involving the ferric hydroperoxo and peroxo intermediates of myoglobin (Garcia-Serres et al. 2007), HRP (Denisov et al. 2002), chloroperoxidase (Denisov et al. 2007), and cytochrome P450 cam (Davydov et al. 2001, Denisov et al. 2001) also failed to provide conclusive spectroscopic evidence of the porphyrin cation radical during the course of annealing. Successes in trapping this elusive enzyme intermediate have been reported in studies using $m$-CPBA as the oxidant. Reactions of cytochromes P450 (Rittle & Green 2010), and to a lesser degree, reactions of a number of myoglobin variants (Matsui et al. 1997, Matsui et al. 1999) with this organic hydroperoxide has led to the accumulation of enough Compound I forms of these enzymes for spectroscopic scrutiny. Although preliminary attempts at following the reaction of $m$-CPBA with IDO1 resulted almost exclusively in the formation of IDO1-Compound II, this result may simply indicate that IDO1-Compound I is comparatively more unstable.

Mechanisms of oxygen insertion into π-bonds by cytochrome P450-Compound I and Compound I model porphyrins are generally believed to be initiated by the formation of a charge-transfer complex between the substrate and the ferryl centre (Ostovic & Bruice 1988, Guengerich & MacDonald 1990, Ostovic & Bruice 1992, Groves & Han 1995, Ortiz de Montellano 1995). The charge-transfer complex has been suggested to react initially by the transfer of one electron from the substrate to Compound I (Ortiz de Montellano et al. 1982, Groves & Watanabe 1986), followed by formation of Fe–O–substrate cation or radical
intermediates, which finally collapse to yield the corresponding epoxide product and Fe$^{3+}$ heme (Guengerich & MacDonald 1990, Sono et al. 1996, Meunier et al. 2004). Oxygen transfer and epoxide formation have also been formulated to proceed in a concerted fashion (Ostovic & Bruice 1992). In principle, the reaction of indole with a putative IDO1-Compound I intermediate can yield indoline-2,3-epoxide and IDO1Fe$^{3+}$ by the above mechanisms (Figure 4.2 A, top pathway). Indoline-2,3-epoxide generated in this manner would spontaneously undergo ring-opening and migration of the hydride to the adjacent carbocation (i.e., NIH shift) to yield the final keto products (i.e., 2- and 3-oxoindole). The apparent regio-selectivities (i.e., approximately 3-fold excess of 2-oxoindole over 3-oxoindole) likely result from additional resonance structures stabilizing the C$_3$ carbocation during the ring-opening step. It should be mentioned, however, that the ratio of products resulting from the ring-opening steps is subject to deviation from the observed product ratio because a fraction of 3-oxoindole is lost to oxidation by O$_2$ during analysis.

On the basis of the steric selectivity observed in the epoxidation of various alkenes by Compound I model complexes, Groves and Nemo have suggested that reasonable geometries for epoxidation of alkenes by cytochrome P450 may have the $\pi$-bond of the substrate approach the iron-oxygen bond from the side in a perpendicular orientation or at an angle $\alpha^2$, as shown in Figure 4.2 B. This geometry is thought to allow favourable interactions between the substrate $\pi$-orbital and the iron-oxygen $\pi$-antibonding orbitals (Groves & Nemo 1983). Interestingly, one possible reason that L-Trp is not readily oxidized by ferryl intermediates of IDO1 is related to the inability of this substrate to adopt this particular transition-state geometry structure as the result

---

[2] Ostović and Bruice examined the epoxidation of various alkenes using a sterically-hindered Fe$^{4+}$=O porphyrin cation radical model catalyst and noted that alkenes need not approach the Fe–O bond in a perpendicular fashion (i.e., $\alpha=0$). Epoxidation can still proceed with alkenes which adopt minimum values of $\alpha$ ranging from 25 to 45° (Ostović and Bruice, 1988).
Fig 4.2 Schematic illustration of indole oxidation by IDO1. (A) Formation of 2-oxoindole by epoxidation (upper pathway) or by direct hydroxylation (lower pathway). (B) Possible orientation of indole with respect to the IDO1 ferryl centre based on the geometries for the epoxidation of olefins proposed by (Groves & Nemo 1983). The curved line represents the approximate contour of the distal heme pocket when viewed straight down the entrance of the active site. The indole ring is plane perpendicular to the porphyrin plane, and its C2–C3 bond is perpendicular to the iron-oxygen bond (left). Favourable overlap between the π*-orbital of the ferryl group and the π-orbital and approaching indole occurs when angle α is minimized. Decreasing angle α results in steric clash between the porphyrin plane and bulky substituents (R) at the C3 position of indole. The Fe$^{3+}$–O$_2$–l-Trp ternary complex (right).
of steric clash of the C₃–derivatized alkyl substituent with the plane of the porphyrin and/or to orientation effects resulting from this substituent. That methyl groups at both C₂ and C₃ (but not at just one of these positions), abrogated oxygen transfer to indole may also be a manifestation of such steric constraints.

Alternatively, oxygen transfer could occur by a hydroxylation mechanism (Figure 4.2 A, lower pathway) that proceeds in two successive one-electron steps involving an indole free-radical and a protonated ferryl intermediate that subsequently recombine to yield the ferric enzyme and oxygenated-indole (i.e., the so-called oxygen rebound mechanism, (Groves & Nemo 1983)). Such a mechanism would result in direct, initial hydroxylation of indole without a discrete epoxide intermediate, followed by tautomerization of the enol product to the final keto-form. In this case, regio-selectivity would be affected by some degree of mobility with respect to the binding of indole at the active-site that results in different orientations of the C₂–H and C₃–H bonds vis-à-vis the ferryl oxygen. Interestingly, though, in the case of methyl-substituted indoles, insertion of oxygen occurred only at the C₂ or C₃ positions adjacent to the methylated carbon but not at the C–H bond of methyl. This apparent selectivity presumably indicates the mechanism by which these indole derivatives are oxidized primarily involves π-attack rather than hydrogen abstraction.

Nevertheless, it is possible that the oxidation of indole by IDO1 involves multiple concurrent pathways that lead to the final keto-products (i.e., 2- and 3-oxoindole). These processes may be deconvoluted through examination of the isotope effects and regio-selectivities, and through correlation analyses (i.e., Hammett analyses). Although the current work suggests involvement of an epoxide in the formation of other indole oxidation products (see section 4.2.4), no direct demonstration of epoxide involvement was possible, so a mechanism involving direct hydroxylation cannot be ruled out definitively. Nevertheless, irrespective of whether the transfer of oxygen occurs by epoxidation or hydroxylation, it is clear
that the near quantitative nature of the transfer must involve a chain of chemical reactions constituting a *bona fide* mechanism typical of oxygenases.

That IDO1-Compound I has not yet been detected during this reaction indicates that the decay of this species to Compound II occurs rapidly. Consequently, capture of the oxygen from IDO1-Compound I by indole must also be prompt. Despite the relatively weak affinity of the Fe$^{2+}$ and Fe$^{2+}$–CO enzyme for indole ($K_d$ 0.5 and 1.5 mM, respectively; (Sono 1989)), rapid-mixing of H$_2$O$_2$ with solutions containing both indole and IDO1Fe$^{3+}$ did not result in significant accumulation of either IDO1-Compound I or Compound II in the pre-equilibrium phase, suggesting that reduction of either ferryl species occurred within the dead-time of the instrument. The disproportionally fast reduction of the ferryl species by indole relative to its millimolar $K_d$ (for IDOFe$^{3+}$) suggests that the change in oxidation state of the heme iron is accompanied by a significant increase in affinity of IDO1 for indole, as is reported for L-Trp (Lu *et al.* 2009, Lu & Yeh 2011). Inhibition of indole oxidation by L-Trp further supports binding of indole at the same general location as L-Trp, but probably with lower affinity.

**4.2.3.c Other possible reactive intermediate of IDO1**

As briefly mentioned earlier, Compound I of cytochrome *c* peroxidase is formally two-electron oxidized from the ferric state like HRP- and cytochrome P450-Compound I, but the second oxidizing equivalent is relocated from the porphyrin $\pi$-system to the protein, typically an aromatic residue (Chance *et al.* 1986, Goodin *et al.* 1986, Huyett *et al.* 1995) (this species is sometimes referred to as Compound ES to differentiate the location of the radical). Although the same is generally true for myoglobin (King & Winfield 1963), whether the protein-centered radical arises directly from heterolytic cleavage of peroxide or by other means remains a point of controversy. While it appears that an IDO1-Compound I is involved in the oxidation of indole, it remains unclear whether a protein based radical is involved as well. While there are known
examples of small molecule oxidation that are initiated by protein based radicals, these processes generally involve dissolved O\textsubscript{2} and occur remotely to the heme. For example, reactions of prostaglandin H synthase, linoleate 8-dioxygenase, and fatty acid \(\alpha\)-dioxygenase involve hydrogen abstraction of their respective substrates by a tyrosyl radical, followed by reaction of the substrate free-radicals with free O\textsubscript{2} (Karthein \textit{et al.} 1988, Hamberg \textit{et al.} 1994, Hamberg \textit{et al.} 2005). Oxidation of linoleic acid to 9-hydroperoxyoctadecadienoic acid by myoglobin (sperm whale) and H\textsubscript{2}O\textsubscript{2} presumably occurs in similar fashion, but in this case, hydrogen abstraction (and O\textsubscript{2} addition) is carried out by the ferryl species rather than the protein radical (Rao \textit{et al.} 1994). On the other hand, olefin oxidation by myoglobin appears to involve both ferryl oxygen transfer and protein-radical mediated O\textsubscript{2} addition (Ortiz de Montellano \& Catalano 1985, Rao \textit{et al.} 1993). In view of the stoichiometric transfer of oxygen atom from H\textsubscript{2}O\textsubscript{2} to indole by IDO1, a scenario involving an aromatic side-chain radical that is located remotely from the heme as described above appears unlikely. For a reaction to proceed in this manner, the protein radical would have to be generated near both the heme and indole groups so that, upon generation of the indolyl radical, this species would be sheltered from dissolved O\textsubscript{2} within the distal heme pocket thereby favouring further reactions with the heme-bound oxygen.

4.2.4 Formation of dioxygenated indole oxidation products

In addition to 2- and 3-oxoindole, a number of more highly oxidized products were observed (\textit{i.e.}, indole-2,3-dione, 2-hydroxy-3-oxoindole, \(\alpha\)-formylaminobenzaldehyde, and indigo blue). Formation of these products is proposed to occur as described in Figure 4.3. Based on the known chemistry of 3-oxoindole, it is likely that once formed, 3-oxoindole can react further with O\textsubscript{2} to form 3-oxoindolenine (VI), which leads to indigo blue through dimerization (Russell \& Kaupp 1969), or to form indole-2,3-dione (III) (Cotson \& Holt 1958). Two
Figure 4.3 Reactions involved in the indole peroxynasge activity of IDO1. Cmpd1 denotes IDO1-Compound 1.
equivalents of IDO1Fe$_{3+}$ can oxidize 3-oxoindole to indigo blue, with the accumulation of IDO1Fe$_{2+}$, presumably as the result of two one-electron oxidations by independent IDO1Fe$_{3+}$. In the presence of H$_2$O$_2$, the Fe$^{4+}$=O/Fe$^{3+}$ couple promotes rapid and extensive oxidation of 3-oxoindole, again presumably by one-electron processes, consistent with the near absence of dioxygenated products derived from anaerobic oxidation of 3-oxoindole by H$_2$O$_2$ and IDO1Fe$_{3+}$.

In contrast, dioxygenated products (indole-2,3-dione (III) and 3-hydroxy-2-oxindole (IV)) can be derived by aerobic oxidation of 2-oxoindole by IDO1Fe$_{3+}$ in the presence of H$_2$O$_2$. The amount of $^{18}$O incorporated into 3-hydroxy-2-oxoindole upon reaction of IDO1Fe$_{3+}$ with H$_2$$^{18}$O$_2$ and indole was consistent with near stoichiometric incorporation of $^{18}$O at C$_2$ and partial (30%) incorporation of $^{18}$O at C$_3$ of this product. While this isotopic distribution could result from the time-dependent exchange of $^{18}$O with H$_2$$^{16}$O, alternative sources for the second oxygen atom are possible (vide infra).

Formation of the o-formylaminobenzaldehyde (an analogue of N-FK) resulting from pyrrole ring opening is notable because this compound is the only product identified here that apparently does not result from the cytochrome P450-catalyzed oxidation of indole (Gillam et al. 1999, Gillam et al. 2000, Gillam & Guengerich 2001, Nakamura et al. 2001, Huang et al. 2007, Li et al. 2008). As noted earlier, however, oxidation of indoles and some indoleamines by HRP can in fact lead to oxidative indole ring opening by the reactions of indolyl radicals with O$_2$ or O$_2$• (Ximenes et al. 2001). In principle, a peroxidatic mechanism could be responsible for IDO1-catalyzed ring opening of indole to yield o-formylaminobenzaldehyde as proposed for the oxidation of melatonin (Ferry et al. 2005), but this possibility is ruled out in the present case based on the incorporation of at least one atom of $^{18}$O from H$_2$$^{18}$O$_2$ into this product. On the other hand, peroxidation could account for IDO1-catalyzed ring opening of 3-methyl-indole and 2,3-dimethyl indole, neither of which proceeded with incorporation of $^{18}$O from H$_2$$^{18}$O$_2$. As IDO1 lacks catalase activity (Shimizu et al. 1978), it is unlikely that sufficient $^{18}$O$_2$ was produced
through disproportionation of $\text{H}_2^{18}\text{O}_2$ to react with a presumed indolyl radical and account for the substantial $^{18}\text{O}$ content in the product. Instead, oxidation of indole to $\text{o}$-formylaminobenzaldehyde and other indole products more likely share a common pathway that is initiated by the transfer of oxygen from IDO-Compound I.

The absence of $\text{o}$-formylaminobenzaldehyde product when 2- or 3-oxoindole were used as substrates implies that neither of these compounds is an intermediate in the formation of the $\text{N}$-FK analogue from indole. Earlier, indoline-2,3-epoxide was proposed to be the initial product resulting from oxygen transfer. Reportedly, indoline-2,3-epoxide is highly labile and decays to the 2- and 3-oxo species (Adam et al. 1993, Skordos et al. 1998), which is consistent with the nearly identical incorporation of $^{18}$O into these two products. As discussed in Chapter 1, the oxidation of L-Trp by IDO1 and TDO has been proposed recently to occur by means of an L-Trp-2,3-epoxide, which subsequently accepts an oxygen atom from the Compound II form of these enzymes to yield $\text{N}$-FK and to regenerate of the ferrous enzymes concomitantly (Basran et al. 2011, Capece et al. 2012). Thus it is possible that regeneration of a ferryl species by a second H$_2$O$_2$ leads to a second oxygen transfer to indoline-2,3-epoxide prior to its re-arrangement to yield $\text{o}$-formylaminobenzaldehyde. Although this product and 3-hydroxy-2-oxoindole incorporated at least one atom of $^{18}$O from H$_2^{18}$O$_2$, sub-stoichiometric incorporation a second $^{18}$O atom was observed (32 and 30 % in $\text{o}$-formylaminobenzaldehyde and 3-hydroxy-2-oxoindole, respectively). Both products exhibited a time-dependent loss of the $^{18}$O through solvent exchange, but the rate at which this loss occurred for 3-hydroxy-3-oxoindole was too slow (30 to 24 % in 3 hr at ambient temperature) to consider exchange as the sole basis for the asymmetric $^{18}$O labeling results. Consequently, additional pathways that involve only one oxygen transfer event in the formation of these dioxygenated indole products are likely. One reasonable alternative involves nucleophilic attack on the epoxide by H$_2$O leading to a vicinal diol (Whalen 1973, Whalen & Ross 1976, Whalen et al. 1977), in this case indoline-2,3-diol. Scrambling of
the second $^{18}\text{O}$ label may result from incorporation of one atom of $^{16}\text{O}$ from bulk solvent or $^{18}\text{O}$ from $\text{H}_2^{18}\text{O}$ that is released from $\text{H}_2^{18}\text{O}_2$ in the active-site after the initial formation of Compound I. Indoline-2,3-diol is suspected to be unstable (Ensley et al. 1983) but could be further oxidized by $\text{H}_2\text{O}_2$ and $\text{IDO1Fe}^{3+}$ to form the stable products $\text{o-formylaminobenzaldehyde}$ or 3-hydroxy-2-oxoindole. The mechanism of this subsequent oxidation remains speculative but may involve cytochrome P450-type ferryl chemistry relevant to C–C bond cleavage of diols (Ortiz de Montellano & De Voss 2004) and alcohol oxidation reactions (Vaz & Coon 1994). Mechanistic details of these reactions remain an active area of discussion, and a number of proposed mechanisms are adapted for indole in Appendix E.

4.2.5 Implications of the indole peroxygenase activity of IDO1

The remarkable selectivity that allows IDO1 to act on indole as a peroxygenase (i.e., $\text{H}_2\text{O}_2$ donor) but on indoleamines exclusively as a dioxygenase (i.e., $\text{O}_2$ donor) underscores the role of the derivatized alkyl substituent in orienting the substrate within the heme cavity, and possibly, in the stabilization of catalytic intermediates at this active-site. As a result, electron transfer from substrate to $\text{IDO1Fe}^{3+}\text{--O}_2\text{--}$ and the resulting cleavage of the $\text{O}\cdots\text{O}$ bond to produce a more highly oxidized form of IDO1 heme iron as believed to occur with L-Trp (Lewis-Ballester et al. 2009, Capece et al. 2010, Yanagisawa et al. 2010, Lu & Yeh 2011, Yanagisawa et al. 2011, Capece et al. 2012) does not occur with indole. Similarly, no appreciable electron transfer from L-Trp bound near the heme iron to the presumed IDO1-Compound I intermediate occurs to initiate substrate oxidation in the peroxygenase reaction. Without more detailed structural or spectroscopic information, it seems likely that the proposed ability of L-Trp to bind to more than one location at the active site of IDO1 is relevant to these observations (Sono 1989, Lu et al. 2009). Specifically, differences in the binding of Trp and indole to secondary sites at the active center of IDO1 may influence the binding orientations of both substrates in proximity to
the heme iron and contribute significantly to the observed catalytic selectivity and the product
distribution.

It is interesting to note that the inability of IDO1 to form Compound I by reductive
cleavage of O₂ in the normal catalytic cycle sets the course to L-Trp oxidation by the
IDO₁Fe³⁺−O₂⋅⁻ intermediate. However, H₂O₂ resulting from auto-oxidation of IDO₁Fe³⁺−O₂⋅⁻,
could enable alternative reaction pathways that involve oxidation of other substrates, including
β-NADH as described earlier and perhaps even putative inhibitors of this enzyme.

While human L-Trp metabolism has been studied in some depth, indole is not one of the
resulting products. Instead, indole is a product of L-Trp degradation by intestinal bacteria that is
absorbed and then metabolized (Vederas et al. 1978, Kawata et al. 1991). Some of the indole
absorbed in this manner is conjugated with glucose to form indican (Dealler et al. 1988), but
indole is also reported to be oxidized by cytochromes P450 as noted earlier. Further work is
required to determine whether indole is oxidized by IDO1 in vivo. Nevertheless, some of the
oxidation products that we have obtained in vitro have well-documented behavioral and
metabolic effects. Isatin and 2-oxoindole for example, are found endogenously in blood, urine,
and cerebrospinal fluid, and have been shown through animal studies to exhibit anxiogenic, anti-
convulsant and sedative effects depending on dosage (Moroni 1999, Medvedev et al. 2005,
Medvedev et al. 2007).

4.3. Biochemical properties of indoleamine 2,3-dioxygenase 2 (IDO2)

4.3.1. L-Trp dioxygenase activity of IDO2

In contrast to IDO1, which was originally discovered as an activity in rabbit intestinal
tissues, IDO2 was discovered through bioinformatic searches of IDO-related sequences in the
human genome database. Because the gene encoding IDO2 lies immediately downstream from
that encoding IDO1 on chromosome 8p12, it has been suggested that IDO1 and IDO2 result from
gene duplication (Ball et al. 2007, Metz et al. 2007). Two transcripts have been reported for the
human IDO2 gene. One transcript is longer and encodes an additional 13 residues at the
N-terminus. This additional sequence is absent in mouse IDO2. The L-Trp dioxygenase activity
of human IDO2 has been demonstrated \textit{in vivo} in transfected T-REx cell (derived from the HEK-
293 human kidney embryonic cell line) and is estimated to be approximately one-half to one-
fourth that of IDO1 based on total cellular N-FK content produced over a period of 48 hr (Metz
\textit{et al.} 2007). However, IDO2 activity appears to be very sensitive to differences in culture
conditions, as similar attempts using HEK-293T cells transfected with either transcripts of
human IDO2 have failed to detect significant N-FK production (Qian \textit{et al.} 2009, Yuasa \textit{et al.}
2010). Although IDO2 mRNA is detected in human dendritic cells and dissected tumors and is
inducible in a variety of human carcinoma cell lines by interferon-\(\gamma\), the enzyme produced
appears to be non-functional when expressed by these cells (Lob \textit{et al.} 2008, Lob \textit{et al.} 2009).
On the other hand, Ball and co-workers have been able to detect significant N-FK production \textit{in vivo}
using mouse IDO2 transfected HEK-293T cells (Ball \textit{et al.} 2007). Interestingly, these
authors note that despite the apparent activity of the enzyme in HEK-293T cells, lysates of HEK-
293T cells expressing mouse IDO2 or \textit{E. coli} recombinant mouse IDO2 had only 3–5\% of the
activity of mouse IDO1 when measured by standard assays \textit{in vitro} (Ball \textit{et al.} 2007, Ball \textit{et al.}
2009).

Human IDO2 has been suggested to be the preferred target of inhibition by the
D-enantiomer of 1MT (D-1MT) when the enzyme is expressed in T-REx cells, whereas L-1MT
primarily targets IDO1 (Metz \textit{et al.} 2007). Uyttenhove and co-workers have shown that pre-
immunized mice bearing IDO-expressing tumors exhibited significant reduction in tumor size
when treated with L-1MT and even greater reduction when treated with D-1MT (Uyttenhove \textit{et
al.} 2003), leading to the proposition that IDO2 also contributes to immunosuppression (Metz
\textit{et al.} 2007). At this time, however, the molecular basis of anti-tumor activity of D-1MT remains ill-
defined. As noted above, IDO2 expressed in human dendritic and tumor cells is not active, so
L-Trp degradation in these cells is mainly supported by IDO1 alone, which in turn is blocked by L-1MT but not D-1MT (Lob et al. 2009). It is possible that the discrepancy in the results obtained from true human malignant cells and IDO2-transfected T-REx cells is related to a common single-nucleotide polymorphism (Arg248 to Trp248) that renders IDO2 inactive in as many as 50% of individuals of European and Asian descent and 25% of individuals of African descent (Metz et al. 2007).

The kinetic parameters of recombinant IDO2 activity that have been obtained in previous reports and the current study are listed in Table 4.1 and 4.2. Although IDO2 appears to retain most of the catalytically important residues and structural motifs identified from mutagenesis and crystallographic studies of IDO1, the enzyme is much less efficient at oxidizing L-Trp than IDO1. The structural basis of this functional discrepancy is unclear, but at least one contributing factor appears to be related to the instability of the recombinant protein. Although several reports have described expression and purification of recombinant IDO2 of various sources (mouse, (Austin et al. 2010); human, (Meininger et al. 2011); vertebrate and fungal, (Yuasa et al. 2010, Yuasa & Ball 2011, Yuasa & Ball 2012)), only one report has included any spectroscopic studies of this enzyme (mouse; (Austin et al. 2010)). These authors noted that the recombinant mouse IDO2Fe³⁺ is found in a mixed spin-state based on the Soret maximum at 406 nm and the α- and β-bands at 538 and 583 nm. However, despite the apparent high-purity that has been achieved in these preparations, the percentage of purified enzyme with heme bound is either low (<27%, (Meininger et al. 2011)) or not reported (Austin et al. 2010, Yuasa et al. 2010, Yuasa & Ball 2011, Yuasa & Ball 2012).

Similar difficulties were encountered with bacterial expression of human wild-type IDO2 in the current work. Specifically, nearly all of the expressed protein was insoluble although a small fraction of each preparation (< 1 mg/L culture) was soluble. The enzyme exhibited well-defined spectroscopic features initially that deteriorated over time becoming obscured by an
Table 4.1 L-Trp oxidation kinetic parameters of recombinant murine IDO1 and IDO2.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Reducing system, assay conditions</td>
<td>Mouse IDO1</td>
<td>Mouse IDO2</td>
<td>Mouse IDO2</td>
</tr>
<tr>
<td>Methylene blue/ascorbate, pH 7.5, 37°C</td>
<td>203</td>
<td>(1.2 \pm 0.1)</td>
<td>(5.5 \pm 0.5)</td>
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<tr>
<td>K_m (µM)</td>
<td>26 ± 4</td>
<td>12,000 ± 3000</td>
<td>530 ± 100</td>
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<tr>
<td>(k_{cat}/K_m) (min⁻¹ µM⁻¹)</td>
<td>7.8</td>
<td>(1.0 \times 10^{-4})</td>
<td>(1.0 \times 10^{-2})</td>
</tr>
</tbody>
</table>

\(a\) Units are converted from µmol product/min/mg enzyme, based on an estimated IDO2 M.W. of 45 kDA.

Table 4.2 L-Trp oxidation kinetic parameters of recombinant human IDO1 and IDO2.

<table>
<thead>
<tr>
<th>IDO isoform</th>
<th>This work</th>
<th>This work</th>
<th>Meininger, D. A., Sutherland, C. L. (2011)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing system, assay conditions</td>
<td>Human IDO1</td>
<td>(a) Human IDO2</td>
<td>(a) Human IDO2</td>
</tr>
<tr>
<td>Methylene blue/ascorbate, pH 6.5, 20°C</td>
<td>111 ± 3</td>
<td>11.0 ± 0.3</td>
<td>10.1 ± 0.1</td>
</tr>
<tr>
<td>K_m (mM)</td>
<td>14 ± 1</td>
<td>3,200 ± 300</td>
<td>410 ± 20</td>
</tr>
<tr>
<td>(k_{cat}/K_m) (min⁻¹ mM⁻¹)</td>
<td>12.4</td>
<td>(3.4 \times 10^{-3})</td>
<td>(2.5 \times 10^{-2})</td>
</tr>
<tr>
<td>(b) Human IDO2</td>
<td></td>
<td></td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Methylene blue/ascorbate, pH 7.0, 37°C</td>
<td></td>
<td></td>
<td>14 ± 4 µM</td>
</tr>
<tr>
<td>(c) Human IDO2</td>
<td></td>
<td></td>
<td>4.3</td>
</tr>
<tr>
<td>Methylene blue/ascorbate, pH 7.0, 37°C</td>
<td></td>
<td></td>
<td>(c) 0.3</td>
</tr>
</tbody>
</table>

\(a\) Variant with 27 N-terminal residues removed (Δ27IDO2).
\(b\) Variant with 13 N-terminal residues removed.
\(c\) Extrapolated values based on the linear relationship of enzymatic activity to [L-Trp].
increase in light-scattering attributed to protein aggregation. These problems were ameliorated to a large extent in the Δ27 variant, which exhibited an electronic absorption spectrum characteristic of a high-spin ferric heme protein similar to the same spectrum of IDO1Fe³⁺. The variant also enabled isolation of IDO2 fully saturated with heme as indicated by the high intensity of the Soret relative to the absorbance at 280 nm \((i.e., 2.2 \text{ for maximal heme incorporation in IDO1})\). As well, a Δ27IDO2Fe³⁺–O₂⁻ derivative could be prepared that exhibited the distinct α- and β-bands characteristic of this form of the protein. However, despite the similarities in the electronic absorption spectra of IDO1 and Δ27IDO2 in their various oxidation and ligation states, the apparent maximal activity of Δ27IDO2 \((k_{\text{cat}} 11.0 \pm 0.3 \text{ min}^{-1})\) was still substantially lower than that of IDO1 \((k_{\text{cat}} 111 \pm 3 \text{ min}^{-1})\).

4.3.2. Factors influencing the dioxygenase activity of IDO2

It has been suggested that the apparent poor reactivity of IDO2 in cell-free assays stems from the lack of suitable reducing systems (Ball et al. 2009, Yuasa et al. 2009, Austin et al. 2010). In the current work, the midpoint potential of the Δ27IDO2Fe³⁺/Fe²⁺ couple, and the half-life for the auto-oxidation of Δ27IDO2Fe³⁺–O₂⁻ to the ferric state (in the absence of L-Trp) were found to be similar to those of IDO1, thereby arguing that reducing conditions sufficient to maintain IDO1 in the fully activated state should be equally capable of supporting maximal IDO2 activity. The inability to produce higher rates of L-Trp oxidation by direct addition of L-Trp to Δ27IDO2Fe³⁺–O₂⁻ is consistent with this expectation insofar as reductive activation of the enzyme is not a rate-limiting factor under these circumstances. Instead, reduced rates of L-Trp oxidation by IDO2 may be attributed to (a) intrinsic low reactivity of His–Fe³⁺–O₂⁻ group (of the active site), and (b) sub-optimal orientation of L-Trp with respect to this catalytic group when involved in the so-called ternary complex.
The positions and relative intensities of the α- and β-bands and the Soret maximum of Δ27IDO2Fe\(^{3+}\)–\(O_2^{•−}\) are nearly identical to those of IDO1Fe\(^{3+}\)–\(O_2^{•−}\). Therefore, it seems unlikely that the electronic structures or reactivities of the His–Fe\(^{3+}\)–\(O_2^{•−}\) centres of the two enzymes would differ substantially, although this assertion remains to be corroborated by additional spectroscopic techniques, particularly by resonance Raman spectroscopy. Addition of L-Trp to Δ27IDO2Fe\(^{3+}\)–\(O_2^{•−}\) resulted in oxidation of the enzyme to the ferric state, so the ternary complex of Δ27IDO2 does not accumulate in sufficiently high levels for spectroscopic comparison to that of IDO1. Nevertheless, cyanide (CN\(^{−}\)) has been used previously as a structural probe to extrapolate L-Trp binding to IDO1Fe\(^{3+}\)–\(O_2^{•−}\) (Lu et al. 2010) given that these two protein species (i.e., IDO1Fe\(^{3+}\)–\(O_2^{•−}\) and IDO1Fe\(^{3+}\)–CN\(^{−}\)) are iso-electronic. Although there are no significant differences in the electronic absorption spectra of Δ27IDO2Fe\(^{3+}\)–CN\(^{−}\) and IDO1Fe\(^{3+}\)–CN\(^{−}\) observed here, spectroscopic changes induced on binding of L-Trp to the two enzymes are significantly different. While binding of L-Trp to human IDO1Fe\(^{3+}\)–CN\(^{−}\) shifts the Soret maximum to higher energy (418 to 416 nm) and decreases its intensity, binding of L-Trp to Δ27IDO2Fe\(^{3+}\)–CN\(^{−}\), in comparison, shifts the Soret maximum to lower energy (418 to 421 nm) with a minimal decrease in intensity. MCD and resonance Raman experiments suggest that the Fe\(^{3+}\)–CN\(^{−}\) group of IDO1 is perturbed in the presence of L-Trp because of steric conflicts resulting from the binding of the substrate immediately adjacent to cyanide (Uchida et al. 1983, Terentis et al. 2002); changes observed in the Soret absorption band are presumably manifestations of these perturbations (Lu et al. 2010). The observation of spectroscopically distinct Fe\(^{3+}\)–CN\(^{−}\)–L-Trp complexes of IDO1 and Δ27IDO2 thus indicate that the orientation of bound L-Trp and its effect on the conformation of heme bound cyanide also differ between the two enzymes. This correlation implies that similar differences in the interactions between O\(_2\) and L-Trp may occur in the respective ternary complexes of IDO1 and Δ27IDO2. Specifically, L-Trp
may bind to $\Delta 27\text{IDO2Fe}^{3+}-\text{O}_2^*$ such that interaction of the L-Trp $\pi$-orbitals with the metal-oxygen orbitals is less favourable for dioxygenase chemistry relative to the situation at the active site of IDO1.

The binding affinities of L-Trp for $\Delta 27\text{IDO2Fe}^{3+}$, IDO1Fe$^{3+}$, and their respective Fe$^{3+}$–CN$^-$ complexes also differ significantly, with $K_d$ values for the ferric and CN$^-$-bound states of $\Delta 27\text{IDO2}$ (7.4 ± 2.1 mM at pH 8.5, and 90 ± 15 µM at pH 7.5, respectively) being at least 26- and 7-fold greater, respectively, than those for IDO1 obtained in the same study. Changes in the electronic absorption spectrum of $\Delta 27\text{IDO2Fe}^{3+}$ on binding of L-Trp are characteristic of OH$^-$ coordination. This high- to low-spin state change is presumably related to substrate inhibition of IDO1 (Sono et al. 1980). Conversely, substrate inhibition in $\Delta 27\text{IDO2}$ is not observed, partly because the low-spin ferric enzyme (i.e., $\Delta 27\text{IDO2Fe}^{3+}$–L-Trp) does not accumulate appreciably at pH 7.5 in the range of [L-Trp] assayed (i.e., up to 15 mM). This observation is also consistent with the proposed differences in the conformation of L-Trp bound to IDO1 and IDO2 insofar as the substrate inhibitory kinetics of IDO1 appears to involve binding of more than one molecule of L-Trp at the active site (Lu et al. 2009), whereas the lack of such kinetic properties in $\Delta 27\text{IDO2}$ suggests that the active-site of this enzyme does not bind a second L-Trp molecule readily.

The apparent affinities ($K_m$) for the oxidation of L-Trp are around 20- to 160-fold lower in $\Delta 27\text{IDO2}$ than in IDO1, depending on assay ingredients (vide infra). Notably, whereas the $K_m$ of IDO1 measured in the presence of methylene blue and ascorbate is approximately equal to the $K_d$ (for L-Trp) of its Fe$^{3+}$–CN$^-$ adduct (14 ± 1 versus 13 ± 3 µM), the $K_m$ of $\Delta 27\text{IDO2}$ measured in the same manner is ~35-fold higher than the $K_d$ of its Fe$^{3+}$–CN$^-$ adduct (3.2 ± 0.3 mM versus 90 ± 15 µM). Other vertebrate IDO2s reportedly exhibit consistently higher $K_m$ values (in the millimolar range) for L-Trp than do the corresponding IDO1 counterparts under similar assay
conditions (Yuasa 2008, 2009) (Table 4.1). These $K_m$ values are much greater than the physiological concentration of L-Trp in tissues and plasma (50–100 µM, (Terness et al. 2007, Torres et al. 2007)). Experiments based on NMR saturation transfer difference spectroscopy provide evidence that methylene blue binds transiently to IDO1 (private communication Moore, G. R). If methylene blue exhibits greater affinity for Δ27IDO2 than for IDO1, then the conditions of the traditional assay could interfere with L-Trp binding to the enzyme and inflate values of $K_m$. This suggestion is consistent with the observation that Δ27IDO2 activity supported by cytochrome $b_5$ and cytochrome $b_5$ reductase coupled system exhibits comparably lower values of $K_m$ (410 µM) probably because the cytochrome $b_5$-Δ27IDO2 interaction does not interfere with L-Trp binding to Δ27IDO2.

4.3.3. IDO2 peroxidase and peroxygenase activities

That IDO2 exhibits peroxidase activity is consistent with its proposed His/H$_2$O coordination structure insofar as H$_2$O should be displaced readily by H$_2$O$_2$. The formation of ferryl derivatives of IDO2 by H$_2$O$_2$ has not yet been characterized kinetically or spectroscopically. Instead, insights into the reactivity of this enzyme with H$_2$O$_2$ are limited to inference from the kinetics of substrate oxidation (i.e., ABTS) catalyzed by activated enzyme intermediates which can be assumed reasonably to be ferryl derivatives of IDO2. Specifically, the Δ27IDO2Fe$^{3+}$-catalyzed peroxidation of ABTS was significantly slower than the reaction catalyzed by IDO1Fe$^{3+}$ for a given [H$_2$O$_2$], which can be interpreted to stem from lower reactivity of Δ27IDO2Fe$^{3+}$ with H$_2$O$_2$, and/or ferryl Δ27IDO2 species with ABTS. As noted briefly in section 4.1, Δ27IDO2Fe$^{3+}$ also reacts with aerobic solutions of β-NADH to form Δ27IDO2Fe$^{3+}$-O$_2^•−$, presumably by the same peroxidase-oxidase reactions described for IDO1. The generality of the peroxidase activity of IDO2 (and IDO1) is unclear given the limited range of substrates explored, but it seems likely that a minimum enzyme-substrate complex that
facilitates electron transfer by IDO2 would have less restricted specificity than that in which oxygen transfer takes place.

As is the case of IDO1Fe$^{3+}$, Δ27IDO2Fe$^{3+}$ catalyzes oxidation of indole by H$_2$O$_2$, but not by O$_2$/O$_2^-$. Similarly, L-Trp is not oxidized appreciably upon activation of Δ27IDO2Fe$^{3+}$ with H$_2$O$_2$. Although structural details concerning the active site of IDO2 are not yet available, it seems reasonable to speculate that indole interacts with this enzyme in a similar manner to IDO1, and that this interaction is also distinct from that of L-Trp, in order to explain the substrate selectivity observed in both IDO enzymes. As proposed for IDO1, it is likely that the oxidation of indole by Δ27IDO2 also involves transfer of oxygen from H$_2$O$_2$ via the putative Compound I equivalent of this enzyme. It should be noted however that the source of oxygen has not been confirmed in the Δ27IDO2-catalyzed reaction, so a peroxidatic mechanism cannot be ruled out at this time. Nonetheless, the specificity constant for the H$_2$O$_2$-dependent oxidation of indole is around 130-fold higher than that for the O$_2$-dependent oxidation of L-Trp by Δ27IDO2 (3.4 ± 0.7 min$^{-1}$ μM$^{-1}$ at 0.5 mM H$_2$O$_2$, and 1.5 ± 0.1 hr$^{-1}$ μM$^{-1}$, respectively) and is in fact close to that of the O$_2$-dependent oxidation of L-Trp by IDO1 (7.9 ± 0.6 min$^{-1}$ μM$^{-1}$). This observation raises intriguing questions regarding the evolutionary relationship of the peroxygenase and dioxygenase functions of IDO enzymes. Phylogenetic analysis of the vertebrate IDO genes suggests that IDO1 and IDO2 arise from gene duplication from a common ancestor which has IDO2-like catalytic properties with respect to L-Trp oxidation (i.e., low $k_{cat}$, high $K_m$ for L-Trp), at some point after divergence of the vertebrate lineage (Yuasa et al. 2009). Therefore it seems that efficient L-Trp dioxygenase activity is acquired during evolution of IDO1, but not IDO2 (also referred to as proto-IDO, (Ball et al. 2009)). It is also interesting to note that IDO-related proteins have been identified among invertebrates (Suzuki & Takagi 1992, Suzuki et al. 1998, Yuasa et al. 2007), but while these proteins have similar gene structures to mammalian IDO
enzymes and bind O₂, they have no detectable dioxygenase activity and are believed in one case to have myoglobin-like functions instead (Suzuki & Takagi 1992, Suzuki et al. 1998). Thus, the wide range of reactivities exhibited by IDO enzymes may be a typical case of functional divergence, whereby the enzymes evolve from their ancestral function as an oxygen carrier to include peroxidase, peroxygenase, and finally dioxygenase functions.

4.4. Concluding remarks

The work presented in this dissertation has evaluated the oxidations of β-NADH and indole catalyzed by IDO1. The first reaction is a peroxidase-oxidase type of reaction which involves one-electron oxidations of β-NADH, and reduction of O₂ without H₂O₂ addition. The second reaction is a peroxygenase reaction characterized by the insertion of oxygen derived from H₂O₂ into the indole C₂–C₃ π-bond. The overall picture that emerges from studies of these reactions is one in which IDO enzymes share common reactivities with heme-containing peroxidases and monooxygenases. As is the case in the reactions catalyzed by these other heme-containing enzymes, both reactions described here presumably involves formation of Fe⁴⁺=O derivatives of IDO (and Fe³⁺–O₂•⁻ in the case of the β-NADH peroxidase-oxidase reaction).

IDO1Fe⁴⁺=O is implicated in the L-Trp dioxygenase reaction and has been detected spectroscopically in previous studies (Lewis-Ballester et al. 2009, Yanagisawa et al. 2010), but the porphyrin cation radical (i.e., Compound I) and protein radical of this species has so far eluded spectroscopic detection. It is possible that additional fine-tuning of the reaction conditions (i.e., peroxide to enzyme ratio, pH, temperature, spin-trap concentrations) can yield detectable levels of these transient species. Variants of myoglobin have been used to stabilize and assist in the detection of the porphyrin cation radical in reactions with peroxides (Matsui et al. 1997, Ozaki et al. 2001), so similar experiments with the IDO enzymes would help in defining the nature of the Fe⁴⁺=O radical in the oxidation of indole. Logically, any variant produced for this purpose
would be evaluated with respect to its ability to oxidize L-Trp. For example, N-FK formation is currently hypothesized to proceed by homolytic O–O bond cleavage from IDO1Fe$^{2+}$–2-indolenylperoxy complex (Capece et al. 2010, Chung et al. 2010). Consequently, variants which promote heterolytic O–O bond cleavage instead may generate L-Trp oxidation side-products and provide additional insights into the mechanisms of oxygen transfer by IDO enzymes. These studies can be further extended to include heme-substitution variants similar to the works of Makino and co-workers on bacterial TDO (Makino et al. 1980). These efforts will ultimately enable a better understanding of the relationships between heme-containing dioxygenases, monooxygenases, and peroxidases.

Because the relative *in vivo* activities of human IDO enzymes vary considerably in reports (Metz et al. 2007, Lob et al. 2008, Ball et al. 2009, Qian et al. 2009), the role of IDO2 in N-FK production and immunosuppression remains unclear. Nonetheless, *in vitro* measurements in previous and current works show IDO2 to be significantly less efficient with respect to L-Trp dioxygenase activity. The molecular basis of the poor dioxygenase activity of IDO2 is not yet rigorously defined. Given that most if not all of the catalytically critical residues identified so far for IDO1 activity are conserved in IDO2, it seems likely that systematic mutagenesis of IDO2 will uncover previously unidentified key residues involved in the oxidation of L-Trp. One such strategy could involve IDO2 variants that emulate the distal heme environment of IDO1. Spectroscopic characterization of the ternary complex of IDO2 also remains undone, due to difficulties in stabilizing this species at ambient temperature. Several strategies have been used in previous studies of the IDO1 ternary complex, such as lowering the temperature to 230 K (Sono 1986, Davydov et al. 2010), or substituting the Fe$^{3+}$–O$_2$$^-$$^*$ species with the inert Fe$^{3+}$–CN$^-$ or Fe$^{2+}$–CO species (Uchida et al. 1983, Terentis et al. 2002, Yanagisawa et al. 2010). Similar experiments could be performed with IDO2 to probe the ligand-substrate interactions of this enzyme. Finally, the crystal structure of IDO2 has yet to be reported, and this information is
essential in order to define substrate and/or inhibitor binding sites. The Δ27IDO2 variant used in this work can be purified to high homogeneity with near full incorporation of heme, and should offer a significant advantage to crystallization over the wild-type enzyme.
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Appendices

Appendix A. DNA sequences of IDO2 and Δ27IDO2 expression constructs

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Appendix B. Sequence alignments of human IDO1 and IDO2 used for homology modeling

Conserved residues are blue. The proximal histidine is shown in green. Segments shown in yellow denote regions of undefined structure in the coordinate set of human IDO1 reported by (Sugimoto et al. 2006).
Appendix C. Derivation of the one-substrate-two-binding-sites kinetic model

Consider the following scheme:

Here, E, S and P denote the free enzyme, substrate, and product, respectively; ES is the enzyme-substrate complex with a single substrate bound; ESS is the enzyme complex formed after binding of a second (identical) substrate. The free enzyme here is considered to be the H₂O₂-activated enzyme instead of the native ferric enzyme, with the underlying assumption that the bimolecular reaction with H₂O₂ is not rate-limiting at high [H₂O₂].

The equations for the ratio of each form of the enzyme (i.e., E, ES, and ESS) to the total enzyme (Et) were constructed using the King-Altman method (King & Altman 1956):

\[
\begin{align*}
(A1) \quad [E]/[E_t] &= \frac{k_2 + k_5 + k_6 + k_3 k_5 + k_3 k_6}{\sum} \\
(A2) \quad [ES]/[E_t] &= \frac{k_1 k_5 + k_1 k_6}{\sum} \\
(A3) \quad [ESS]/[E_t] &= \frac{k_1 k_4}{\sum}
\end{align*}
\]

The denominator is the sum of the numerator in the three equations (A1 to A3). The rate equation for product formation for the model under consideration is:

\[
(A4) \quad \frac{d[P]}{dt} = k_3 [ES] + k_6 [ESS]
\]

Substitution of [ES] and [ESS] by equations A2 and A3 yields the following mathematical model:

\[
(A5) \quad \frac{v}{[E_t]} = \frac{k_1 k_3 k_5 + k_1 k_3 k_6 + k_1 k_4 k_6}{k_1 k_4 + k_1 k_5 + k_1 k_6 + k_2 k_5 + k_2 k_6 + k_3 k_5 + k_3 k_6}
\]

In order to account for substrate concentration, the term [S] is multiplied in every step that contains the rate constants for substrate binding (i.e., k₁ and k₄) to yield the following chemical model:

\[
(A6) \quad \frac{v}{[E_t]} = \frac{k_1 k_3 k_5 [S] + k_1 k_3 k_6 [S] + k_1 k_4 k_6 [S]^2}{k_1 k_4 [S]^2 + k_1 k_5 [S] + k_1 k_6 [S] + k_2 k_5 + k_2 k_6 + k_3 k_5 + k_3 k_6}
\]
Equation A6 can be simplified by defining \((k_2+k_3)/k_1\) and \((k_5+k_6)/k_4\) as \(K_{m1}\) and \(K_{m2}\), respectively, and by denoting \(k_3\) and \(k_6\) as \(k_{\text{cat1}}\) and \(k_{\text{cat2}}\), respectively, the following form is obtained:

\[
(A7) \quad v = \frac{k_{\text{cat1}}[E][S] + \left(\frac{k_{\text{cat2}}[E][S]^2}{K_{m2}}\right)}{\frac{[S]^2}{K_{m2}} + [S] + 1}
\]

If \(K_{m2} \gg K_{m1}\) or \([S]\), that is the saturation of the enzyme by one substrate occurs at low substrate concentration whereas saturation by a second molecule of the substrate occurs only at very high substrate concentrations, equation A7 can be reduced to the form obtained by Korzekwa and co-workers (Korzekwa et al. 1998) that is used in section 3.2.8:

\[
(A8) \quad v = \frac{k_{\text{cat1}}[E][S] + \left(\frac{k_{\text{cat2}}[E][S]^2}{K_{m2}}\right)}{K_{m1} + [S]}
\]

If \(k_{\text{cat2}} \approx 0\), that is the saturation of the enzyme by a second molecule of the substrate fully inhibits enzyme activity, then equation A7 takes on the form of the classic steady-state model of substrate inhibition (A10) used in section 3.3.6, where \(K_{m2}/K_{m1}\) corresponds to the substrate inhibition constant \(K_{si}\):

\[
(A9) \quad v = \frac{k_{\text{cat1}}[E][S]}{\frac{[S]^2}{K_{m2}} + [S] + K_{m1}}
\]

\[
(A10) \quad v = \frac{k_{\text{cat}}[E][S]}{\frac{[S]^2}{K_{si}} + [S] + K_m}
\]
Appendix D. Calculation of percent $^{18}$O incorporation

The relative abundances of [M+H]$^+$ ions were corrected for natural abundance contributions using the methods reported by (Mirgorodskaya et al. 2000).

For example, consider the natural abundance contributions for $\sigma$-formylaminobenzaldehyde derived from natural H$_2$O$_2$ results in a series of [M+H]$^+$ ions of m/z 150 through 156, with relative abundances of [100, 9.9, 1, 0.1, 0, 0, 0] for the seven ions, respectively (black columns).

The incorporation of one or two atoms of $^{18}$O results in a shift of +2 and +4 m/z, respectively. The calculated isotopic distribution for a homogeneous singly $^{18}$O or doubly $^{18}$O labeled species are [0, 0, 100, 9.9, 1, 0.1, 0] (hashed columns) and [0, 0, 0, 0, 100, 9.9, 1] (cross columns), respectively. The three series comprise matrix $A_{(3x7)}$.

The observed isotopic relative abundances, [19.9, 4.4, 100, 10.4, 58.4, 3.8, 0.3] is a sum of the three contributing species (shown above as equal contributions for illustrative purpose) and is regarded as matrix $B_{(1x7)}$.

The relative ratio of each species, vector $X_{(3x1)}$, is then solved by least-squares approximation by the equation $X = (AA^T)^{-1}AB^T$, resulting in the ratios of 0.12, 0.56, and 0.32 for the $^{16}$O$^{16}$O, $^{16}$O$^{18}$O, and $^{18}$O$^{18}$O species, respectively.

Finally, H$_2$O$_2$ used in this work is $\sim$90\% $^{18}$O enriched. Random distribution of the isotope at either one or both of two oxygen positions of $\sigma$-formylaminobenzaldehyde produces a calculated ratio of 0.9 and 0.81 for the $^{16}$O$^{18}$O and $^{18}$O$^{18}$O species, respectively, assuming fully stoichiometric transfer of the isotope. The observed ratio of $^{16}$O$^{18}$O and $^{18}$O$^{18}$O, normalized with respect to the theoretical ratio of transfer, are therefore 0.62 and 0.40, respectively.
Appendix E. Alternative reaction routes for dioxygenated indole oxidation products

Nucleophilic attack of the indoline-2,3-epoxide by water results in the formation of indoline-2,3-diol, which reacts with IDO1-Compound I resulting in (A) C$_2$–C$_3$ bond cleavage to o-formylaminobenzaldehyde (reaction scheme based on proposals in (Meunier et al. 2004)), and (B) oxidation to 3-hydroxy-2-oxoindole (reaction scheme based on proposals in (Bell-Parikh & Guengerich 1999))